DISSERTATION ON

ALTERATION IN APPARENTLY NORMAL BUCCAL MUCOSAL CELLS OF SMOKERS AND NONSMOKERS USING SILVER STAINING NUCLEOLAR ORGANISING REGIONS

A STUDY OF 75 CASES

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CONTENTS

ABBREVATIONS

Ag+	-	SILVER ION
AgNOR	-	Silver stained nucleolar organizing regions
DNA	-	Deoxyribonucleic Acid
D.P.X	-	Dibutyl phathalate xylene
EGFR	-	Epidermal Growth Factor Receptor 2
GST	-	Glutathione-S-transferases
HIV	-	Human Immunodeficiency Virus
ICD	-	International Classification of Diseases
mAgNOR	-	Mean AgNOR
NDMA	-	Nitrosodimethylamine
NNK	-	Nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
NNN	-	Nitrosonornicotine
NORAP	-	Nucleolar organizing region associated protein

OSCC	-	Oral Squamous cell carcinoma
PAH	-	Polycyclic aromatic hydrocarbons
p-value	-	Probable value
rDNA	-	Ribosomal DNA
RNA	-	Ribonucleic acid
SD	-	Standard deviation
UGTs	-	Uridine-5′-Disphosphateglucuronosyltransferases
WHO	-	World Health Organisation

ABSTRACT

BACKGROUND: Smoking is considered to be initiators of dysplastic changes in the oral mucosa.

AIM: The aim of this study was to determine and compare the alteration in apparently normal buccal mucosal cells in smokers and non –smokers due to the effect of tobacco by assessing silver stained nucleolar organiser regions. (AgNOR)

MATERIALS AND METHODS: The study comprised of 75 subjects divided into two groups with 25 subjects having smoking habit and 50 subjects who were non smokers. Cytological smears were taken from each subject with the help of a cytological brush. The smear was wet fixed and stained with AgNOR and assessed for nucleolar organising regions.50 cells were counted in each slide.

RESULTS: Unpaired T-test and Pearson's R correlation test was applied. Cytological changes in smokers revealed an increase in mean AgNOR in comparison with non-smokers.

CONCLUSION: Tobacco smoking produce alterations in apparently normal buccal mucosal cells, which may cumulatively lead to carcinomatous changes. Results of these changes may be used as an educational tool in cessation of smoking in the general population.

INTRODUCTION

Medical science has made considerable progress with respect to infectious diseases. In case of carcinoma, there is a lot to be achieved, although a good deal of innovations and lifesaving therapies have been discovered. In case of oral cancers, it begins with use of tobacco. It is the powdered leaf of a plant which was used in a Y shaped piece of cone called “Tobago”.

Malignancy of the oral cavity is the sixth most common malignancy worldwide.[1] It has a dismal 5-year survival, except when it is detected in early stages. The established method for diagnosis is by biopsy, which is carried out only when patient is symptomatic. Hence it is of little value in detecting at an early stage and preventing the progression.

In Indian subcontinent, oral cancer is one of the most common forms of cancer. Mostly they are squamous cell carcinomas. It is a major public health problem. The habit of tobacco smoking is associated with leukoplakia. As oral cancers arise from premalignant lesions, the effect of early screening reduces the risk for oral cancers.

The prognosis is good if detected earlier, in case of mouth cancers. Many methods have been used to identify pre-malignant lesions as a marker of impending malignancy. They are assessment of mitosis, DNA ploidy status, DNA and RNA in situ hybridization, monoclonal antibodies to detect proliferation related antigens. The issue is, they are expensive, time consuming and need sophisticated equipment.

Nucleolar organizing regions are loops of DNA, that transcribe to the ribosomal RNA, which in turn results in synthesis of proteins by the cell. Nucleolar organizing regions are correlated with cellular proliferation.

AgNOR staining is an inexpensive and easy method for identifying nucleolar organizing region with this idea, our present study has been conducted to assess the accuracy of AgNOR as a proliferation marker in buccal smear.

“Doctors, nurses, midwives, dentists, pharmacists, chiropractitioners, psychologists and all other professionals dedicated to health can help people change their behavior. They are on the frontline of tobacco epidemic and collectively speak to millions of people.”

Quote by DR.Lee jong-wook,

Former Director General, WHO(2005)

AIM OF THE STUDY

To determine and compare the alteration in apparently normal buccal mucosal cells in smokers and non -smokers due to the effect of tobacco by assessing silver stained nucleolar organizer regions (AgNOR)

REVIEW OF LITERATURE

ILL EFFECTS OF SMOKING

People can be broadly classified as non -smokers and smokers .Further,they are subclassified as

- Non smokers - a) Never smokers
  b) Former smokers
- Smokers - a) Minimal-who smoke <15cigarettes / day
  b) Moderate-who smoke between 15-25cigarettes / day
  c) Heavy –who smoke >25 cigarettes / day[2]

They are prone to a wide range of diseases. Smoking can lead to recurrent pulmonary infections, COPD, pulmonary tuberculosis, peripheral vascular diseases, myocardial infarction, stroke, hip fracture. It can have a major role in the etiopathogenesis of various cancers. In 20th century it killed about 100 million People worldwide.

There is a well-known correlation between smoking and cancer of various organs like lung, bladder, oral cavity, larynx.

MECHANISM

The cancer causation by cigarette smoking follows these steps

(1) Exposure to the carcinogens.

(2) Establishment of bonds between carcinogens and DNA.

(3) Accumulation of somatic mutations in genes.

Each puff of cigarette has many compounds, of which 60 are well known carcinogens. These carcinogens can be subdivided into various classes, including, aromatic amines, volatile organic compounds, polycyclic aromatic hydrocarbons (PAH). The metabolites of these carcinogens are elevated in blood, breath and mainly urine.[3]

Most carcinogens undergo a metabolic activation process and change them to metabolites which attach to DNA. This results in production of DNA adducts. Metabolic activation of cigarette carcinogen requires P-450s 1A2, 2A13. The induction of P-450 is critical for cancer susceptibility in smokers[4]. Metabolic detoxification is carried out by enzymes like uridine-5′-disphosphate-glucuronosyltransferases (UGTs), glutathione-S-transferases (GSTs). The equation between

detoxification and activation of carcinogens shows considerable variation among persons and affects susceptibility to malignancies.

The carcinogens, once activated results in DNA adduct production, which is vital in the carcinogenic process. A few can directly form DNA adducts. Studies found that adduct levels is elevated in smokers .[5]

The repair system removes DNA adducts. They are direct repair of DNA bases by alkyl transferases, double-strand break repair, and mismatch repair[6]. If these repair enzymes do not function properly. These adducts may remain and raise the chance of producing somatic mutations.

Persistent DNA adducts miscoding during replication of DNA. Particular DNA adducts leads to specific somatic mutations. KRAS and Tp53 oncogenes are subjected to such changes.

Gene mutations results in a change which disturbs the normal proliferation of cells and leads to cancer. The phenomenon of apoptosis counteract against these mutational events and eliminating DNA which are damaged. The balance between process of apoptosis and events suppressing apoptosis plays an important role in tumor proliferation.

Nicotinic receptors are the sites where nicotine and nitrosamines bind. These activate protein kinase A and protein kinase B[7]. Smoking stimulates production of EGFR[8] and COX-2, which results in changes leading to cell proliferation. These products enhance the carcinogenicity of these constituents of tobacco. The changes which carry an increased risk of cancer, are reverted on cessation of smoking.

An important pathway of enzymatic hyper methylation, which is termed as epigenetic changes[9]. It produces gene silencing of mainly tumor suppressor genes and can lead to proliferation of cells which are unregulated.

CARCINOGEN EXPOSURE

The exposure of smokers to these carcinogenic compounds is 1.4 to 2.2 milligrams per cigarette. *N*-nitrosamine and aromatic amines, PAHs are a few carcinogens which are quiet potent and less in quantity. Acetaldehyde and isoprene are higher in quantity, but their potency is quiet less.

PAHs are incomplete combustion products. Some PAHs such as benzo[*a*]pyrene (B (a)P) have a very high ability to produce malignancies.[10]

Similar to PAH are heterocyclic compounds and they contain nitrogen. one of the compound, which is a liver carcinogen is furan.[10]

A group of potent carcinogens are N-nitrosoamines. (methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and *N'*-nitrosonornicotine (NNN) are two of them.[10]

Aromatic amines are products that include 4-aminobiphenyl (4-ABP) and 2-naphthylamine which were first recognized as bladder carcinogens due to exposure to various dyes[10].

The volatile hydrocarbons are 1,3-butadiene and benzene, a known leukemogen. 1,3-butadiene and benzene are potent carcinogens in cigarette smoke[10].

Other carcinogens in cigarette smoke include vinyl chloride and ethylene oxide. Ethylene oxide is linked to lymphatic and hematopoietic malignancies. Cigarette also houses few metals like cadmium,polonium.[10]

URINE , BREATH AND BLOOD BIOMARKERS

The carcinogens or their metabolites can be quantified by analyzing urine, breath, and blood. The products whose measurement is made in urine are

1. Trans, Anti-Phet and 1-HOP for PAH[11]

2. Total NNAL (NNAL plus NNAL glucuronides) for NNK

3. MHBMA for 1,3-butadiene;

4. tt-MA , S-PMA for benzene.

Smokers show more amount of the products Benzene, 1,3-butadiene in expired air.

Benzene and styrene, are elevated when measured in the blood of smokers.

Cotinine is a metabolite of nicotine. Cotinine assay is useful to estimate smoking in adults and smoke exposure in non smokers and children.

Metabolism of various carcinogens of cigarette smoke

The metabolic activation convert substances in cigarette smoke causing cancer to intermediate products. The products

interact with nucleophilic site in DNA, the ensuing product being DNA adducts. Detoxification reactions compete with metabolic activation. The DNA adducts B[*a*]P-7,8-diol-9,10-epoxides (BPDEs)[12] are potent mutagen which are produced following activation of B[*a*]P. The BPDE formation involves

1. Metabolism of B[*a*]P to B[*a*] P-7,8-epoxide.
2. Hydration of B[*a*]P-7,8-epoxide resulting in dihydrodiol B[*a*]P-7,8-diol.
3. More epoxidation to form BPDE.

One enantiomer reacts with DNA to form adducts at N^2 of deoxyguanosine and found to be highly carcinogenic.

Detoxification pathways compete with B[*a*]P metabolic activation by forming phenols through direct hydroxylation of epoxides and Dihydrodiols by hydration of epoxides formation of glutathione, glucuronide, and sulfate conjugates..

An Unstable α-hydroxymethyl metabolite forms on activation of NDMA by α-hydroxylation. Methanediazohydroxide is a product obtained when this unstable compound loses formaldehyde. It is also one of the products of NNK. Denitrosation is the way detoxification is carried out with production of nitrite

and methylamine. The protein forms cross-links and other products as it interacts with formaldehyde, an aldehyde produced by NNK.[13]

α-hydroxylation of NNN produces an intermediate which leads to pyridyloxobutyl (POB)-DNA adducts. Denitrosation produces norcotinine which is the way NNN is detoxified[13].

7-(2-hydroxyethyl) guanine is a product as ethylene oxide interacts with DNA. Glutathione conjugation and excretion of mercapturic acids is the pathway for detoxification.

Benzene and 1,3-butadiene undergo activation during their metabolism

Benzene is converted to benzene epoxide, on interaction with DNA produces 7-phenylguanine[13].

Metabolism of 1, 3-butadiene is through epoxidation and form monoepoxide that further changes to products, which results in DNA adduct formation. Important among these are dihydrodiol epoxide which results in cross-links in DNA[13].

Enzy mes in Carcinogen Metabolism

Enzymes are essential in the activation and detoxification of carcinogens in cigarette. Few of them are P-450s, *N*-acetyl-

transferases (NATs), epoxide hydrolases, and sulfotransferases GSTs, UGTs. Their involvement in the metabolism of a carcinogen depends on properties of carcinogen properties like lipophilicity, size, polarity and of the property of enzymes which are regulation of expression structure and tissue distribution.

Cytochrome P-450 Enzymes Cytochrome P-450 Enzymes

P-450s are microsomal enzymes which involve oxidation of carcinogens. The enzymes P-450s 1A1 and 1B1 are the ones which catalyse the metabolism of PAH. Aromatic amines are processed with the help of the enzyme P-450 1A2.

P-450s 2A6, 2A13 and 2E1 are involved in the sequence of events leading to formation of metabolites from N-nitrosamines [14]. P-450 2E1 is the key enzyme in the process of metabolisation of benzene and 1,3-butadiene which involves the process of epoxidation. P-450 1A2 is the catalyst for aromatic amine *N*-oxidation. Smoking induces and increases the levels of this enzyme in the liver. .

Glutathione-S-Transferases(GST)

The detoxification of epoxides which exhibit carcinogenic potential ,is conjugation with glutathione. The cytosolic GSTs are the enzymes involved as the catalysts of glutathione conjugation of

the carcinogens. These four GST classes to which the enzyme belong are: alpha (GSTA1-1), mu (GSTM1-1), pi (GSTP1-1), and theta (GSTT1-1)[15].

GSTM1-1 and GSTT1-1 are vital for the metabolism of two 1,3-butadiene epoxide metabolites which involves conjugation. The two metabolites mentioned are 3,4-epoxybutene (EB) and diepoxybutane. The glutathione conjugation of benzene oxide produces S –PMA. This is the way by which benzene is detoxified and excreted in urine. GSTM1-1 or GSTT1-1 are involved in the conjugation of benzene oxide .

Ethylene oxide is detoxified by glutathione conjugation. GSTT1 acting as a catalyst of this reaction in smokers expressed increased level of 2-hydroxyethylvaline Hb adducts. The carcinogens involved are ethylene and ethylene oxide in cigarette smoke.

Uridine-5'-Diphosphate-Glucuronosyltransferases

Various carcinogens undergo metabolisation by conjugation with glucoronic acid. The microsomal enzyme Uridine- 5-diphosphate - glucoronyl transferases are part of the process. They are divided into two groups (UGT1 and UGT2). The diol and Phenol metabolites of PAHs are converted to glucuronide conjugates and

later eliminated. The excretion of the NNN is done after conjugation involving UGT. Detoxification of benzene is by conversion into products formed after conjugation with glucoronic acid.[13]

Acetyltransferases

Aromatic amines undergo N-acetylation ,which is the process by which they are eliminated. *N*-hydroxy metabolites of arylamines generated by P-450 (e.g., *N*-hydroxy-4-ABP), goes through a process of *O*-acetylation is activates them and forms DNA Adducts[13]

DNA Adducts and Biomarkers

HPLC, liquid chromatography, electrochemical detection, ^{32}P-postlabeling and immunoassay are various methods to analyze DNA adducts.

Adducts and sources are

1. 1.7-methyldeoxyguanosine - NDMA, NNK,
2. O6-ethyl-deoxyguanosine and O4-ethylthymidine- ethylating agent (chemically uncharacterized).
3. pyridyloxobutylate DNA-NNK and NNN
4. 7-(2-hydroxyethyl) deoxyguanosine –Ethylene oxide

5. N6-ethenodeoxy-adenosine, 3, N4-ethenodeoxycytidine - vinyl chloride , ethyl carbamate.

Some studies document clear evidence for elevated levels of adducts resulting from exposure to specific carcinogens such as B[*a*]P, NNK, or NNN[16]. .

Carcinogen-albumin and carcinogen-Hb adducts are used to indirectly estimate DNA adducts. Advantages of Hb adducts as surrogates are easy accessibility of Hb in blood and the lifespan of red blood cells allowing adequate time to cumulatively aggregate in sufficient quantities for detection.[16]

The Hb adducts of aromatic amines are biomarkers acting as a good source of information on the effects of smoking. Levels of these adducts are higher in smokers than in nonsmokers, particularly for 3-ABP–Hb and 4-ABP–Hb adducts. Adduct levels show a reduction on cessation of smoking and are related to the number of cigarettes smoked. The amino terminal valine of Hb is the site where adducts attach and provide useful information .Smokers show increased Ethylated N-terminal valine of HB on comparison with nonsmokers.

Smokers show higher DNA adducts. DNA adducts are classified into

1. Nonspecific adducts-detected by immunoassay and ^{32}P-postlabeling
2. Specific adducts, which are detected by structure-specific methods. .

Measuring levels of Hb adducts is very simple way to assess carcinogen exposure of the cell. Accumulation of DNA adducts leads to genetic damage. The propagation of this genetic damage during clonal outgrowth is consistent with the accumulation of multiple genetic changes observed in cancer progression.

Conversion of DNA Adducts to Mutations

DNA replication has an vital role to play in producing point mutations through insertions or deletions. DNA adducts are not by itself mutations .It can be deleted by repair mechanisms present in DNA. The ability to serve as a template is hindered when there is a modification in the form of deletion or insertion of a base. Therefore, DNA synthesis slows down or is blocked at the site of the adducted template.

Gene promoter hyper methylation can result in loss of gene transcription and gene function is silenced. In initial stages of tumor formation, P16 gene undergoes this process of hyper methylation. AGT promoter hyper methylation, affects *TP53* gene and results in tumor proliferation.[17]

Nicotine or NNK promotes rapid multiplication of cancer cells and new blood vessel formation. Cell-surface receptors and further cytoplasmic kinase activation is carried out by carcinogens present in smoke Among them, the two most important ones are proteins in BCL-2 family [18]and NF-κB. These components also play a role in suppressing apoptosis. Genetic and epigenetic events can lead to cancer causation. Critical cellular pathways are involved in proliferation of these transformed cells.

Genetic polymorphisms

Genetic polymorphisms have a role to play in tobacco-related neoplasm. Cigarette smoking is definitely a major risk factor in the development of cancer at various sites. It is a fact that smokers develop cancer. It is also further noted that not all smokers turn into patients harboring malignancies. This variation has kindled interest in genetic polymorphisms and carcinogen-metabolizing enzymes.

Polymorphisms in phase I and II enzymes have been observed. Phase I enzyme result in oxidation of the carcinogen, while phase II enzymes convert the carcinogen into products which are easily excreted. These products are predominantly water soluble. The enzymes which are catalyst for this reaction are seen to play a role in the activation and detoxification of carcinogens. Studies of the autopsy shows polymorphisms of *CYP1A1* and *GSTM1* and a raised DNA adduct levels. It can lead to minor and major variations in the metabolic pathways .This can be assumed as the cause of variation in response to carcinogens in smokers. Many studies have extensively investigated whether variation in metabolizing carcinogens can lead to variation in lung cancer risk.[19]

Cytochrome-P1A1 Gene (CYP1A1)

Inter individual variations in the ability to activate carcinogens such as PAH through the *CYP1A1* gene leads to differential carcinogenic effects. Studies say that there are two variant polymorphisms in the *CYP1A1* gene. The first is a base change in intron 6, which results in a new *MSPI* restriction site. The other polymorphism is in exon 7, a base change which results in change in amino acid .

Among patients with squamous cell carcinoma (SCC), the homozygous variant genotype was associated with an increased risk of developing lung cancer, especially in those with a lower cumulative dose of cigarette smoke. .

Studies have also associated the *ILE462VAL* polymorphism of *CYP1A1* with lung cancer risk in Japanese and Chinese populations.[20] Again, the homozygous variant **VAL/*VAL* genotype was associated with lung cancer at lower cumulative doses of cigarette smoke. The effects of genetic variability and differential enzymatic activity are more apparent at low doses, when saturation has not been reached.

A study of African Americans and Mexican Americans showed a twofold increase in the risk of lung cancer among light smokers with the *MSPI* variant genotype. However, a Brazilian study showed an increase in risk with the **ILE/*VAL* polymorphism but not with the *MSPI* polymorphism.

Researchers found an association in Whites between the *CYP1A1* homozygous **MSPI* variant and lung cancer risk after adjustment of values for age and gender.

CytochromeP2E1 Gene (CP2E1)

The *CYP2E1* gene plays a role in the activation of NDMA, as well as other carcinogens. Le Marchand and colleagues made case-control study with 341 lung cancer cases and 456 controls. These researchers found that *CYP2E1* polymorphisms were associated with a decrease in risk of lung adenocarcinoma. A Chinese study confirmed this finding. However, the presence of at least one variant *CYP1A1 *MSPI* allele was associated with an increased risk of SCC. Associations between *CYP1A1* and *CYP2E1* polymorphisms and subsets of lung cancer indicate PAHs induce SCC and nitrosamines induce adenocarcinomas.[21]

CytochromeP2A13 Gene (CYP2A13)

The *CYP2A13* gene plays a role in the metabolic activation of *N*-nitrosamines such as NNK. Researchers have identified a polymorphism in *CYP2A13* in which a amino acid substitution is seen at position 257. The variant 257CYS protein, the product of this gene, has one-third the capacity of the 257ARG protein to activate NNK. In a study of 724 lung cancer patients and 791 control Observed that this variant of *CYP2A13* genotype was correlated with a lower risk for lung cancer, adenocarcinomas in

specific. The reduction in risk did not reach statistical significance for SCC or other histologies of lung cancer[22].

Gluthatione S-TransferaseM1 Gene (GSTM1)

Large variations in enzymatic activity for several GSTs are seen. The GSTM1 enzyme is important in detoxifying carcinogens.

Some studies have found a link between the *GSTM1* null mutation and lung cancer across many populations. In a study in Japan, the *GSTM1* null genotype positively correlated with Squamous Cell Carcinoma of the lung, not with adenocarcinomas. An analysis in a Finnish population also associated the *GSTM1* null genotype with SCC. A U.S. study claimed that *GSTM1* null genotype association showed modest elevation in lung cancer risk, which increased among heavy smokers.[23] With stratification by the amount of smoking, the proportion of *GSTM1* null genotype increased progressively in the SCC group from 50 percent in light smokers to 72 percent in heavy smokers. One study made a conclusion that higher intakes of cruciferous vegetables reduced lung cancer risk among persons with the *GSTM1* genotype. However, several other studies have shown a greater protective effect in persons with the *GSTM1* null genotype who consumed cruciferous vegetables [24].

The effect of *GSTM1* appears to be increased by gene-environment and gene-diet interactions. The high frequency of *GSTM1* polymorphisms observed across all ethnicities may contribute to the importance of this variant as a risk factor for developing lung cancer.

CYP1A1 and GSTM1 in Combination

Studies of the effect of combined *CYP1A1* and *GSTM1* variant genotypes hypothesized that increased PAH activation and decreased PAH detoxification in tobacco smokers might lead to an increase in lung cancer risk. Numerous studies have explored this association.

Combination of the *CYP1A1* variant genotype and the *GSTM1* null genotype enhanced the risk of smoking-related lung cancers in a Japanese population. Hayashi and colleagues (1992). They found a raise in frequency of the homozygous **VAL/*VAL* genotype combined with the *GSTM1* null genotype in lung cancer patients . The cigarette dose is low then *CYP1A1* and *GSTM1* may be an important determinant of susceptibility to cancer [25]

Persons with these variant genotypes in both *CYP1A1* and *GSTM1* had a much higher risk of lung cancer than did those with

the variant *CYP1A1* and wild-type *GSTM1* . Studies in Scandinavian populations ,see an increase in the risk of lung cancer with the combination of variant *CYP1A1* and *GSTM1* genotypes.

Gluthatione S-Transferase P1 Gene (GSTP1)

Polymorphisms in the *GSTP1* gene is associated with family of phase II enzyme. One *GSTP1* polymorphism includes an base change that leads to an isoleucine→valine substitution, which results in lower enzymatic activity toward 1-chloro-2,4-dinitrobenzene but higher activity toward PAH diol epoxides. In the largest study with 1,042 cases and 1,161 controls, the *GSTP1* homozygous variant genotype was associated with a higher lung cancer risk at any level of exposure to smoke than was the wild-type genotype.[26]

In a study of 1,694 cases and 1,694 controls, double variants in *GSTM1* and *GSTP1*, as well as in *GSTP1* and *TP53*, were associated with an increase in lung cancer.

Gluthatione S-Transferase T1 Gene (GSTT1)

In a study of Chinese living in Hong Kong, the *GSTT1* null genotype was associated with a higher risk of lung cancer than was the functional *GSTT1* genotype only in non-smokers. A study from

Denmark also suggested that the *GSTT1* null genotype is associated with a higher risk of lung cancer.[27]

N-Acetyl Transferase 2 Gene (NAT2)

Polymorphisms of the *NAT2* gene are linked with decreased activity or reduced stability of the enzyme. The polymorphisms result in slow or fast acetylation. Most studies report no overall increase in risk with the genotype for either slow or fast acetylation. A study involving 1,115 lung cancer patients and 1,250 control participants, *NAT2* genotype and lung cancer risk exhibited no association. It showed significant interaction with smoking. Among nonsmokers, the genotype for rapid acetylation decreased lung cancer risk more than slow acetylation. In smokers persons with the genotype for rapid acetylation had a higher risk. A research from Taiwan said that the *NAT2* genotype for fast acetylation is associated with an increased risk of lung cancer among women who were never smokers.

The NAT2 protein is involved in bio-activation and detoxification of the aromatic amines associated with cigarette smoke.

The risk of cancer was raised in smokers and higher chances among them were for persons with slow acetylation.

The *NAT2* genotype for slow acetylation is associated with an increased risk of bladder cancer, cigarette smoking and occupational exposure to aromatic amines showing cumulative effects[28].

Microsomal Epoxide Hydrolase (MEH)

MEH acts as an activator and a detoxifier of carcinogens. MEH is the enzyme which hydrolyses epoxide intermediate agents and converts them to less reactive dihydrodiols which is eliminated. MEH metabolizes PAH epoxides and activates them. Several identified polymorphisms include a base change in exon 3 leading to an amino substitution at residue 113, which involves a decrease in enzymatic activity. A base change in exon 4, which involves substitution at residue 139, which shows increase in enzymatic activity[30]. There is a positive correlation between polymorphism causing an increase in enzymatic activity and risk of developing lung cancer. A study found this to be true with young Mexican Americans having exon 4 polymorphism, had an increased risk.. The presence of exon 4 and exon 3 polymorphisms, exhibited high enzymatic activity and also an increased risk . In a study in Taiwan, high MEH activity**,** recognized by combination of exon 3 and exon

4 polymorphisms, was associated with an increased risk for SCC (Lin et al. 2000).

In nonsmokers, MEH activity is inversely proportional to cancer risk. It is due to inability to counter the effect of environmental pollutants by MEH when in low doses. The adenocarcinomas of the lung has better prognosis if exon3 polymorphism is present. This shows Exon3 polymorphism has a protective role to play.

CONCLUSIONS ON THE MECHANISM OF CARCINOGENESIS IN SMOKERS

1. The doses of cigarette smoke carcinogens are indicated by the level of metabolites in Blood, Urine.
2. DNA adducts are formed by the carcinogens which undergo metabolic activation which is induced by CYP-450
3. In smokers, carcinogens can cause numerous changes at cellular level, which is caused by direct effect of these products.
4. Cigarette smoke carcinogens can lead to mutations in *TP53* and *KRAS* .

5. Smoking is associated with methylation of genes like *P16, which are tumor suppressors* in lung cancer and other cancers The smoke constituents such as nicotine and 4-(methylnitrosamino) -1-(3-pyridyl)-1-butanone permit the survival of damaged cells. It is carried out by means of activation of signal transduction pathways.

6. Smoking cessation is the only means of achieving the target of stopping the progress of pathogenic processes leading to cancer

ANATOMY OF ORAL CAVITY

Oral cavity is defined as the region from vermilion junction of the lips to the line of the circumvallate papillae of the tongue below and junction of the hard and soft palate above (Fig. 1). It can be divided into eight areas:

1. Lip mucosa
2. Buccal mucosa
3. Lower alveolar ridge
4. Upper alveolar ridge
5. Retromolar gingival (RMT)
6. Floor of mouth (FOM)
7. Hard palate
8. Anterior two-thirds of tongue(the OT)

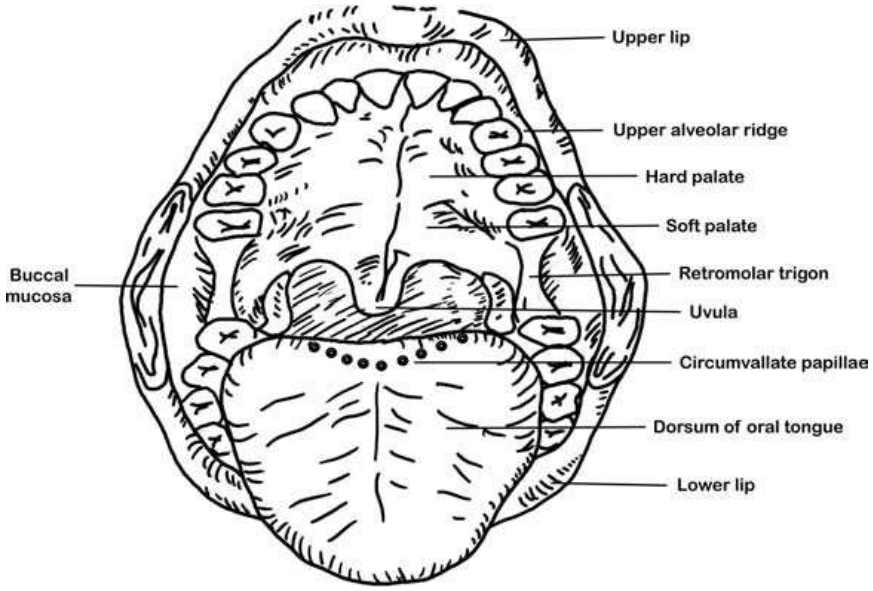


FIG-1 **ANATOMY OF THE ORAL CAVITY**

EXAMINATION OF THE ORAL CAVITY

A simple clinical examination of the oral cavity is useful in identifying few premalignant and malignant lesion .Studies say that changes in buccal cells offers promise as biomarkers for early detection of oral cancer.

The investigations can be classified as in vivo and ex-vivo

The in vivo tests differentiates normal, precancerous and cancerous epithelial cells.It is done using novel imaging techniques like fluorescence spectroscopy[30],photodynamic imaging of tumors that have incorporated photofrin[31] .The other in vivo test is to identify high risk premalignant lesion using toluidine blue[32]. It has been used successfully in clinical studies which correlated with histological progression.

Ex-vivo tests include biopsies of normal and malignant tissues, scraping containing exfoliated buccal cells have been in vogue for quiet sometime[33]. Tests to identify buccal cell changes will be adjunct to clinical examination of oral cavity of subjects.

HISTORY OF EVOLUTION OF ORAL CYTOLOGY

The Papanicolaou and Traut studied the cells from cervical mucosa for precancerous and cancerous lesion paved way for oral

cytology. This work proved an effective tool for screening malignant disease of the cervix [34] .In the initial stages, comparative studies of oral and cervical cytology was done .They observed cellular changes based on the phase of menstrual cycle.

In late nineteenth century, sputum yielded malignant cells indicative of oropharyngeal carcinoma[35]. The diagnosis of nasopharyngeal carcinoma was done by using papanicolou staining in oral smears. . Cytology of the oral cavity was considered useful by many researchers. Later, Sandler, by his major work named Veterans Administration studies of oral cytology, opined that this technique helps in early detection of oral malignancies[37].

Important events in oral exfoliative cytology

1860	- Beale	-	sputum in a case of pharyngeal carcinoma showed malignant cells
1940	- Weinmann	-	Cytological examination of oral cellular keratinisation
1941	- Ziskin et al.	-	Effects of the menstrual cycle on oral cellular morphology
1942	- Papanicolaou	-	Introduction of a staining procedure for cytological smears

1943 - Papanicolaou and Traut - Cytological diagnosis of uterine cancer

1949 - Morrison et al. - Cytological diagnosis of nasopharyngeal malignancies

More recent advances in oral cytology are

Meyer, Rubinstein and Medak (1970)[34]

Mucosa of the smokers and non-smokers was compared. In both the groups, subjects had clinically healthy mucosa and they found early response to smoking is in the form of change to less keratinized cells. Meyer *et al* suggested that exfoliative cytology in smokers is a useful investigation to identify alteration in apparently normal buccal mucosa.

Bernstein and Miller (1978)[35]

They stated oral exfoliative cytology, if properly used could be a tool for the early identification of premalignant and malignant lesions. Advantages of the cytology techniques despite its pitfalls, is simple, painless, inexpensive and rapid

Hillman and Kissin (1980) [36]

Studied exfoliative cytologic smears from the cheeks of 790 alcoholics and evaluated the correlation between microscopic

features and selected indicators of nutritional status and observed that a significant association existed between the cell/nucleus ratios. They observed that poor diet patterns caused an increase in nuclear size and reduction in cell/nucleus ratio.

Cowpe JG and Longmore RB, (1981) [37]

Made a morphometric analysis on clinically normal buccal mucosal cells with parameters like nuclear area .There was no significant variation seen in the nuclear size.

Van Molengraft et al (1982)[38]

Observed that there was no significant difference in cellular and nuclear diameters between solitary and clustered cells.

Scott J et al (1983)[39]

They said a reduction in nucleus/cytoplasmic ratio with advancing age in the study on normal lingual epithelium.

Cowpe et al (1984)[40]

They used quantitative techniques to assess the exfoliative cytology smear from normal and abnormal human oral mucosal. The study noticed that the malignant lesions exhibited abnormal DNA distribution histograms and the cytomorphological values displayed significant variation, when compared with the normal squames.

Cowpe JG and Semmens HE, (1985) [41]

Nuclear and cell size of normal buccal squamous cells are un affected by menstrual cycle. It came to a conclusion that oral smears do not demonstrate the time of ovulation or stage of menstrual cycle

Burkhardt et al (1985) [42]

The success in exfoliative cytology in early detection of cervical cancer has motivated numerous studies on oral mucosa. But unlike the uterine cervix, metaplasia does not occur in the oral cavity. It is also observed that only superficial cells are recovered which gives little information about the deeper layers and makes its value very restricted in the oral cavity.

Cowpe, Longmore and Green (1985) [43]

Oral smears were obtained from various sites of the oral cavity, of different age groups and of both sexes. There was significant variation in cytoplasmic area, nuclear area and at different sites .The nuclear size varied significantly with increasing age. It also stated there were no differences between males and females.

Smeulders and Dorst (1985) [44]

A sharp definition of the objects to be measured or not to be measured using strict qualitative criteria is therefore essential. The poor quality of slide preparation can cause difficulty in identifying the objects to be measured. Moreover, poor fixation resulting in ballooning and vague outlines and under staining or over staining hinders the segmentation (discrimination between foreground and background). Optimizing staining techniques and the quality of slides is of major importance in quantitative cytopathology.

Abdel Salam et al (1986) [45]

Studied the utility of image analysis in distinguishing among oral white lesions. They observed that image cytometry is a upcoming field of research for oral disease. The analysis of morphology of cells and nuclear parameter is useful in understanding behaviour of the disease.

Hill and Gibson (1987) [46]

Their study was on the oral and dental effects of Qat chewing in 121 volunteers and found that the buccal mucosa in 50% of the subjects was normal and in the remaining subjects some degree of keratosis was found and none showed any evidence of malignancy or dysplasia

Creath, Shelton and Wright (1988) [48]

Studied the prevalence of use of smokeless tobacco by adolescent athletes. The associated abnormal mucosal findings were noted. They observed that oral leukoplakia is more common in those who had dipped smokeless tobacco for more than 2 years would have a high incidence of oral leukoplakia.

Cowpe, Longmore and Green (1988) [49]

Applied quantitative techniques to the smears collected from the abnormal buccal mucosa and floor of the mouth, which were compared with normal smears. In their study, statistically significant variations were observed in nuclear and cytoplasmic areas .The abnormal smears showed an increase in the nuclear area and a reduction in cytoplasmic area. The result displays a good success rate for identifying premalignant and malignant lesions .The quantitation provides an excellent investigation to detect early oral malignancy.

Chen S.Y. (1989) [50]

Experiments on male & female rats in which their buccal mucosa was subjected to application of tobacco on the buccal mucosa for 1 year. It established that the tobacco treatment can disturb the mitotic process

Bramlye and Smith (1990) [51]

There are methods other than biopsy such as exfoliative cytology for sampling oral cancer. They stated that it is a very simple procedure. The disadvantage is many false negative results, mainly of the precancerous lesions of oral mucosa.

Ogden, Cowpe and Green (1990) [52]

The study made an effort to use semi-automated image analysis techniques on smears obtained from normal oral mucosa .The regions which were suspected of being dysplastic were assessed for the cytomorphologic features and DNA content of buccal mucosal cells .It was found that smears with malignancy had a significant reduction in cell area, in comparison with normal subjects

Gao S et al, (1995) [53]

The study attempted a Morphometric analysis on the spinous cell of oral sub mucous fibrosis and compared it with normal oral mucosa, leukoplakia, dysplasia and carcinoma. Surface area, perimeters, and all kind of diameters of the cells showed a progressive decrease from normal mucosa through dysplasia to carcinoma. The nuclear cytoplasmic ratio showed a progressive increase. The dimensions of nuclei did not show any significant differences.

Sugerman P.B. and Savage N. W (1996) [54]

They said oral cytology is a non-invasive procedure to assess changes in oral epithelium and its utility is in the screening for dysplasia and carcinoma. They concluded that exfoliative cytology is possible in patients abusing tobacco in the form of betel quid, who can develop oral malignancies. The smears from premalignant and malignant lesions exhibited a raised nuclear cytoplasmic ratio, increased keratinization, and pleomorphism of the nucleus.

Gray T, Bancroft J D, Stevens A (1996) [90]

Cytomorphology of oral cavity made significant progress with digital image analysis. Digital image processing systems use images held in a computer's memory. This allows the computer to look at an image correctly and opens the possibility of fully automatic analysis of the image by a computer programme. The digital video image is divided into an array of small rectangles, called pixels and their appropriate brightness value is stored in the computer's memory. Multiple objects, consisting of groups of numerous pixels, can be automatically selected and measured if the brightness value is sufficiently different from the surrounding background. The process normally involves the production of the temporary coloured binary image that is areas to be measured are

coloured red while the rest are coloured blue. This is superimposed over the digitized image so that the operator can check which objects are to be measured. This can be fixed so that automatic measuring is possible. To display the digital image, each pixel's memory location is read and redisplayed on the monitor.

Colour selection is performed by defining the unique ratio of red, green and blue for a specific colour, which can be re-expressed as its hue, saturation and intensity (HIS).

T.Ramesh et al (1998) [55]

They studied the changes in morphology based on cell diameters and nuclear diameter of squamous cells of oral premalignant and malignant lesions. They suggested that nuclear diameter and Cell Diameter could be utilised in the investigations to identify oral lesions which are premalignant and malignant.

Ogden, Wright and Rice (1999) [56]

The study analysed the ways alcohol affects quantitative cytomorphometry on the oral mucosa. There was reduction in the mean nuclear area and mean cytoplasmic area in alcohol group. This could be due to dehydrating action of alcohol on the cells.

Heloisa de Castro Sampaio et al (1999) [57]

Compared the AgNOR count of cells collected from normal buccal mucosa of cigarette smokers with that obtained from non-smokers. Cytological smears of normal buccal mucosa from 20 smokers and 20 non-smokers were stained for AgNORs. The AgNOR count was established in 100 cells. The mean AgNOR count was higher in cells of smokers than non-smokers.

Amit Chattopadhyay et al (2002) [58]

Silver stainable nucleolar organizer regions (AgNORs), as proliferative marker may play a role in identifying dysplasia in tissue specimens. Mean AgNOR is a good parameter for defining objective parameters in dysplasia

Cytology Techniques and Modifications

Over years, as oral cytology grew, many investigators experienced the limitations of oral cytology and felt the need for improvements. They introduced many modifications which procured larger amount of cells, to sample a large cellular area and improve the quality of cell staining. Special stains have been advised, to identify the best area for cell collection in a diffuse lesion .The cells of the basal and Para basal layers can be visualized, if the atypical

keratotic cell layers are removed.Few advocated the use of a metal spatula or a sharp spoon for this procedure.

Numerous analytical methods for light microscopy were used. The use of fluorescence microscopy and phase contrast microscopy was explored. Fluorescent DNA-specific dyes like Acridine Orange measure the cellular DNA content . Cytomorphological parameters for malignancy, was done with the help of image cytometry. Besides these applications of the oral cytological studies, Epstein–Barr virus can be demonstrated in hairy leukoplakia. Oral cells can be obtained from a sample of saliva,material from rinsing or by scrapping of the mucosa

The instrument for making a nice cytological smear must show the following features

1. Must be at ease to use even at difficult sites in the mouth

2. Traumatic damage must be very minimal

3. Help in procuring adequate number of cells

Brush biopsy is a simple, safe highly sensitive, relatively cheaper method of screening for cancer. A full Trans epithelial cellular sample is procured with ease with the brush .The

combination of smears and image analysis system go a long way in recognizing oral epithelial abnormalities. Pinpoint bleeding is evidence that a full thickness sample has been obtained.

The introduction of the oral brush is important in the history of oral cytology. Use of a brush for cervical cytology demonstrated better cell spreading on objective slides as well as an improvement in quality and validity of smears compared with smears obtained by using a wooden spatula[59]. It is a more convenient instrument, than the wooden tongue depressor, in oral lesions. This technique is painless and easy investigation that can be used to assess any suspicious lesion.

A study stressed the need for brush biopsy with automated imaging, where clinically benign-appearing mucosal lesions were sampled using this technique and 5% were later confirmed by biopsy to represent dysplastic epithelial changes or invasive cancer. Many others opined that brush cytology could discover lesions that were not clinically suspicious of carcinoma or pre-invasive disease . A study clubbed conventional oral brush cytology and application of toluidine blue to identify the site for brushing in suspicious mucosal regions. Mehrotra et al.,in his study recently, impressed on the utility of automated analysis in minimally suspicious lesions.

Evolution and Modifications in techniqes in oral exfoliative cytology-

1951 - Gladstone - Improved quantities of obtained cells by use of a “sponge biopsy”

1952 - Schneider - Modifications of staining

1960 - Cawson - Modifications of staining

1963 - King - Use of frosted glass slides

1963 - Staats and Goldsby- Comparison of wooden and metal spatula.Recommendation of the metal spatula

1964 - Sandler - Removal of keratotic layers with a sharp Curette

1981 - Dumbach et al. - Smear curettage’. Inclusion of deeper cell layers by use of a curette

Few recent advances in methodology in oral exfoliative cytology are

Ogden G R et al (1989)

Assessed the effect of fixatives on morphological features in oral cytology. Three methods were employed. These included spray fixation,Direct immersion in ether and ethanol in equal proportions

and air drying. The observation was no difference found , whatever fixation method used.

Van Diest et al (1989) [61]

It investigated mechanical influences of the smear and of preparation techniques on cells and nuclei. Neither method led to an area dependent distribution (area gradient) of the cells or nuclei on the side of induced orientation of the cells or nuclei on the slide or induced orientation of the cells or nuclei.

Herzberg A J, Raso D S, Silver man J F (1999)[62]

In his color atlas of normal cytology, used number of techniques in the collection of oral epithelial cells for cytologic examination. Instrument like wooden tongue depressors, metal spatulas, and cotton tipped applicators were commonly used.

Oral cytology is vital in the early diagnosis of oral cancers, as a technique for obtaining cell samples that is analysed by sophisticated diagnostic techniques such as DNA cytometry, and molecular analyses and cytomorphometry. The techniques like Toluidine blue staining, brush biopsy and sophisticated computer programs have changed the field of oral cytology. It has made interpretation of findings far more reliable. The cytological study of oral cavity is simple, non-aggressive and relatively painless, and

rapid. It is accepted by patients and suitable for application in population screening programmes, for early analysis of suspect lesions, and for pre-and post-treatment monitoring of confirmed malignant lesions.

The oral cavity is squamous epithelial lining with difference in surface keratinization at various sites.

SAMPLING METHODS OF THE ORAL CAVITY

The mouth is sampled by smears obtained by scraping. The scrape smears are obtained with a tongue depressor or a small curette. The lesions covered with thick layers of keratin, require a more vigorous scraping with a sharp metallic instrument .

INDICATIONS FOR CYTOLOGIC EXAMINATION

The application of cytologic techniques to the oral cavity is the diagnosis of occult carcinomas, not identified or not suspected on clinical inspection.

CYTOLOGY OF NORMAL ORAL SQUAMOUS EPITHELIUM

Squamous Epithelial Cells

Normal squamous epithelium of the oral cavity has superficial and intermediate squamous cells. They occur singly or in clusters and are identical with squamous cells that are found in specimens of sputum and of saliva .

Condensation of the nuclear chromatin like a nuclear bar with lateral extensions, has been recorded in superficial squamous cells by Wood et al (1975)[63]. These cells are seen in the mucosal surface of the adjacent floor of the mouth and the lower lip in healthy people . The change is probably related to “nuclear creases” . Similar cells may be observed in mesothelial cells in the pericardium, surface of the conjunctiva and in other organs.

Superficial cells without nuclei are known as keratinized squames, more common from the palate and are normal. All stages of transition between nonkeratinized and keratinized cells may be observed. Smaller para basal squamous cells are seen, if an ulceration is present or procedure is done vigorously. Squamous oral cells carry blood group on their membranes.[64]

Other Cells

The nasopharynx and salivary gland duct produces columnar cells rich in mucin. Lymphocytes, singly or in clusters are seen, when base of tongue or tonsillar area is vigorously scraped.

Oral Flora

Entamoeba gingivalis is fairly common. People who have poor oral hygiene harbor many types of bacteria and saprophytic fungi. An unusual organism, Simonsiella species, was described by Greene Baum et al in smears of oropharynx, sputum, and gastric aspirates in 1988[65]. The large bacteria form caterpillar-like chains, made of 10 to 12 individual bacteria. The bacterial chains are readily observed overlying squamous cells (Fig. 21-2B). The organism is nonpathogenic, is observed in mouths of people with rich dietary intake, particularly fat and proteins.

Buccal Squamous Cells in Genetic Counseling and as a Source of DNA

Buccal smears are the cheapest and easiest-to-use test to determine genetic sex, by observing and counting sex chromatin (Barr bodies) in squamous oral cells. The Barr bodies are half-moon shaped chromatin condensation at the nuclear membrane. In genetic females all squamous cells with nonpyknotic, open vesicular nuclei should contain a Barr body.In reality, it can be

identified in fewer than half of these cells by light microscopy of oral smears stained with Papanicolaou's stain. Peripherally placed chromocenters and focal thickening of the nuclear membrane may mimic Barr bodies. Occasionally, malignant cells may contain two or more Barr bodies, reflecting aneuploidy.[66]

The presence of Barr bodies in a phenotypic male strongly suggests Klinefelter's syndrome (47 chromosomes, YXX). The absence of Barr bodies in a phenotypic female suggests Turner's syndrome.

Buccal cells collected by mouthwash or by other techniques acts as a source of DNA for various tests, including person identification.

INFLAMMATORY DISORDERS

The diffuse inflammatory processes and poor oral hygiene produces ulceration or erosions. The large Para basal squamous cells come into picture than the superficial and intermediate cells which are normally seen. They exhibit is multiple, round or oval vesicular nuclei. Small nucleoli and chromo centers are noted. The cytoplasm is often poorly preserved.

Multinucleated macrophages may occur in chronic inflammatory process. These conditions express purulent exudate and leukocytes. Plasma cells are also seen, more commonly with smears obtained from posterior pharynx

SPECIFIC INFLAMMATORY DISORDERS

Oral Herpes

This common disorder, characterized by blisters and painful ulcerations, is caused by Herpes virus type 1. Kobayashi et al (1998) observed the pathognomonic cell changes in smears of only 4 of 11 patients in whom the diagnosis could be confirmed by culture.

Moniliasis (Thrush)

Clinically, moniliasis forms a characteristic white coating of the oral cavity. This organism may be identified by finding the characteristic fungal spores and pseudohyphae. It occurs in diabetics, HIV patients. In HIV patients, it may be the first clinical symptom.

CHANGES IN ORAL SQUAMOUS CELLS IN DEFICIENCY DISEASES

In deficiencies of vitamin B_{12} and folic acid, both the nucleus and the cytoplasm of the squamous cell show increase in size. Megaloblastic anemia, tropical sprue may show identical changes[67]. Vitamin B_{12} and folic acid are vital ingredients for DNA synthesis. If either one is inadequate, the DNA synthesis is altered, exhibiting cell enlargement.

OTHER BENIGN DISORDERS

Benign Leukoplakia

Heavy keratin formation on the surface of epithelium is a process localized to areas like the palate, parts of gingiva. The benign leukoplakia is milky white appears histologically as a benign squamous epithelium, with layers of keratin. The precancerous leukoplakia may have a similar clinical appearance.

Cytology of benign leukoplakia is fully keratinized, yellow stained cells without nuclei being characteristic of this disorder[68]. It may also be seen in normal oral smears; therefore, the cytologic diagnosis should always be correlated with clinical findings.

MALIGNANT LESIONS

Invasive Squamous Carcinoma and Its Precursors

RISK FACTORS

Abuse of alcohol and more importantly smoking are the key epidemiologic factors in patients developing malignancies of the mouth. Tobacco contains carcinogens which initiates and promotes cancers in the oral cavity. Tobacco in any form, such as pipe-, cigar-, or cigarette smoking, reverse smokers (people holding the burning end of a cigarette in their mouth), betel-nut chewers (tobacco powder is often wrapped inside the betel leaf) represent high-risk populations. The latter two forms of tobacco use are seen mainly in India and other parts of Southeast Asia. In America, African-Americans have a higher risk of oral squamous cancer than other people (Skarsgard et al, 2000).

Pure ethanol is never carcinogenic, but increased risk of upper aero digestive tract cancer is associated with alcohol drinking in non-smokers. Alcohol acts as a solvent, facilitating the package of carcinogens through cellular membranes which has a role in carcinogenesis.

Acetaldehyde produced by microflora by the oxidation of ethanol is responsible for alcohol associated carcinogenesis.

Several studies have reported association between poor oral hygiene and oral cancer. Experimental evidence in animals show localization of chemical carcinogens induced tumors to the sites of repeated mucosal traumatization.

Clinical observations in human cases describe carcinomas developing at sites of chronic trauma caused by a broken teeth or ill-fitting dentures. It is certain that the role of inflammation, due to poor oral hygiene, is seen in the pathogenesis of oral carcinoma. It is compounded by known carcinogens such as tobacco, alcohol abuse, nutritional deficiencies.

Dietary deficiencies of Vitamin A, C, and E are associated with oral cancers.Trace elements such as zinc, selenium may contribute to development of oral cancer.A study in china found a strong protective effect of carotenoid, vit.c and fiber intake in oral cancer risk.

An increased risk for oral cancers is HIV /AIDS population and immunosuppression in organ transplant recipient.The incidence of cancer of lowerlip is increased in both population

Human papillomavirus (HPV) presence in oral cancer has been suspected for some years and has now been documented in lesions of the oral cavity[69]. Garelick and Taichman (1991) observed HPV types 2, 4, 6, 11, 13, and 32 in the benign lesions, including leukoplakia, and HPV types 16 and 18 in oral carcinomas. Paz et al (1997) observed HPV sequences in only 15% of squamous cancers of the esophagus and the head and neck area. HPV was mainly observed in tumors of the tonsillar area and in some metastases. The presence of HPV had no prognostic significance. Mork et al (2001) considered infection with HPV type 16 as a risk factor in squamous cancer of the head and neck.

Cytology

The biopsy of clinically suspicious lesions yields a diagnosis of invasive squamous cell carcinoma. The ulcerated invasive lesions can be diagnosed cytologically. It is necessary to remove necrotic material before cytologic sampling. The background always shows blood, numerous leukocytes and necrotic material.

The degree of keratinization can be assessed by cytology. The cancer cells are large withorange- and yellow-staining cytoplasm. They also have pyknotic, dark-staining irregular nuclei. “Ghost” cells have no nuclear material. The mean AgNOR value of a

squamous cell carcinoma is the highest in comparision with benign and precursor lesions. The cytologic diagnosis of nonulcerated, invasive, keratinizing carcinomas, like verrucous carcinoma , is not clear as abundant “ghost” cells hinder the observation of malignant cells. Reddy and Kameswari (1974) made a study with 165 patients with keratinizing carcinoma of the hard palate in reverse smokers in India and diagnosis was made in only 60% of the patients.[70] Similar results were reported by Bànóczy and Rigó (1976). A detailed observation of nuclear abnormalities was made, which occurs in only a few cells. Irregularity of outline, nuclear hyperchromasia, nuclear enlargement, is of diagnostic significance. A biopsy is advised if diagnosis is suspicious.

In poorly differentiated squamous carcinomas, keratinization is not prominent, , but coarse chromatin and nucleoli which is large is seen. In oral cancer, nucleocytoplasmic ratio is usually modified in favor of the nucleus

PRECURSOR LESIONS IN ORAL SQUAMOUS CELL CARCINOMA

Identifying precursor lesions may be lifesaving in case of oral cancers. Precancerous lesions of the squamous epithelium of origin invariably precede invasive cancer.

Two types of precancerous lesions in the oral cavity:

The common white lesions with irregular, jagged borders, usually referred to as precancerous leukoplakias, similar to the benign leukoplakias, and correspond to precancerous lesions with a heavily keratinized surface and nuclear abnormalities in well-differentiated squamous cells . The white color of the lesion is due to the opaque surface layer of keratinized epithelium. Mild or Moderate dysplasia is often attached to such lesions.[71]

- The less common red lesions (erythroplakia), corresponding to the nonkeratinizing precursor epithelial lesions, are usually composed of smaller cancer cells with minimal or absent keratinization of surface (carcinomas in situ or severe dysplasia) [71]. The red color is because of vascularized stroma underlying the often thin epithelium. The lesion is a precursor of invasive squamous cancer .It is recognized in the studies by Sandler (1962, 1963), Shafer et al (1975), and Mashberg et al (1977). Niebel and Chomet (1964) suggested in vivo staining of the oral mucosa with toluidine blue to demarcate the territories of these lesions.

- Incidentally discovered, there are no visible oral lesions. Acetowhite areas, after application of 3% acetic acid solutions, may be observed in such patients.

It is difficult to diagnose precancerous leukoplakia and keratinising carcinoma in situ.The abnormal cells in smears are obscured by anucleated squames or keratinized benign cells. Few visualized cells suggest either a borderline squamous lesion or a well-differentiated squamous cancer with keratinized cytoplasm and nuclear enlargement. In these conditions, it is of prime importance to note the clinical finding .The information from cytology may be minimal, is a indication to proceed for a biopsy

Hong et al opined that a beneficial effect on the size and degree of cellular abnormalities in oral precancerous leukoplakias of some patients is seen with administration of 13-cis retinoic acid.

Nonkeratinizing Lesions

Oral carcinoma in situ or severe dysplasia, are not similar to precancerous leukoplakia. They are seen without significant keratin formation on their surfaces and have malignant epithelial cells. All these lesions present clinically as areas of redness (erythroplakia) .

Scrape smears from such lesions are characterized by the presence of both malignant parabasal and intermediate cells with marked nuclear enlargement, hyperchromasia with a translucent cytoplasm. A few squamous cancer cells show marked nuclear

abnormalityand keratinized cytoplasm. The smear pattern in oral carcinoma in situ is remarkably similar to that of a high-grade squamous precursor lesion of carcinoma of the uterine cervix of well-differentiated type.

Stahl et al (1964), observed the implications of dyskaryosis in oral mucosal lesions, pointed out the necessity of long-term follow-up of patients showing such cells in their smears.

RESULTS OF CYTOLOGIC SCREENING FOR OCCULT CARCINOMA AND PRECURSOR LESIONS

The difficulty in clinical identification of precancerous leukoplakia and carcinoma in situ, both easily curable precursor stages of oral cancer, was appreciated in an extensive cytologic study of mouth lesions which was conducted by the Veterans Administration, guided by Dr. H. Sandler. There were 2,758 patients with visible mouth lesions identified by cytology, and there were 287 histologically documented cases of invasive carcinoma. Many of these lesions were very small, many were not ulcerated, not indurated, and not fixed to the underlying tissue.

There were 28 patients with carcinoma in situ. Thirteen lesions were reddish in color, 6 were white, and the rest were of various colors; only 6 were ulcerated and only 5 indurated.

Redness of circumscribed areas of oral epithelium, erythroplakia is frequently characteristic of carcinoma in situ.

- Shafer studied the clinical and histologic data on 82 oral carcinomas in situ diagnosed by biopsy only. The comparison of clinical findings of both the studies showed roughly 50%of Sandler's lesions were red, whereas there were only 16% of such lesions in Shafer's survey, suggesting that even competent observers consider red oral lesions as benign and do not biopsy them. Such lesions should be the prime target for cytologic screening.[72]

A survey by Stahl et al (1967) confirmed that cytologic screening for oral cancer is feasible. It does not appear feasible or reasonable to cytologically screen all dental patients. A scrape smear of an oral lesion may well permit more conservative surgery for earlier lesions and may be life saving.AgNOR count in precursor lesion is on the higher side in comparison with benign lesions. Shiboski et al (2000) recently made an observation of deficiency in the education of professional and public education for early diagnosis of oral cancer.

Sandler's, Shafer's, and Mashberg and Meyer's studies pointed out that the floor of the mouth was the most frequently affected site of oral squamous cancer, followed by lateral surface of tongue and soft palate. These areas deserve a careful inspection during routine dental examination.

Within recent years, there has been a revival of interest in cytologic detection of oral cancers based on evaluation of oral smears by a semi automated cell analysis system OralCDx (Sciubba, 1999). A specially designed brush was used to secure cell samples from the visible lesions of the oral cavity. Of the 945 lesions sampled by cytology, 131 were “dysplastic” lesions or carcinomas confirmed by biopsies. In these cases, the smears were judged to be either “positive” or “atypical” .

Extensive surveys are necessary,in which increased risk of oral cancer exists. One such study by Wahi, impressed upon the value of cytologic techniques among betel-nut chewers. The table gives details of the study.The data strongly suggest that high-risk candidates for oral cancer are primary target for screening by cytologic techniques for oral malignancies.

TABLE 1 CYTOLOGIC DIAGNOSIS OF ORAL CARCINOMA AMONG BETEL-NUT CHEWERS

Total cases of oral cancer studied	812
Clinically unsuspected (66 squamous carcinoma, 2 reticulum cell sarcoma, 1 adenocarcinoma)	69
Clinical diagnoses on 69 unsuspected cases	
Leukoplakia	26
Ulceration	27
Trismus	9
Dysphagia	4
Tonsillar enlargement	3
Cytologic diagnoses on the same cases	
Malignant cells	39
Cells suggestive of cancer	21
Dyskaryotic cells, possibly Malignant (Prof. P.N. Wahi, Agra, India, personal communication.)	9

CYTOLOGIC DIAGNOSIS OF RECURRENT ORAL CANCER AFTER TREATMENT

- A close follow-up of all patients is essential when local recurrences after treatment is possible. The have an increased chance of treated patients to have a second malignancy or a recurrence. The addition of cytologic techniques with the follow-up examination may result in the diagnosis of a recurrent or new cancer ,before it is suspected clinically.[73]

- Hutter and Gerold (1966) used cytologic techniques in the follow-up of patients previously treated by surgery. The application of cytology to the patients without visible lesions, uncovered clinically unsuspected recurrent cancer in 10 of 177 patients investigated. They used material scraped from the general area of prior surgery by an endometrial curette.

- This work, as well as the results of cancer detection surveys described above, strongly suggest that the silent stage of carcinoma in situ, whether primary or recurrent, is not readily identifiable clinically and precedes invasive squamous carcinoma of the oral cavity. This stage of cancer may last for several months, and possibly much longer, before producing a visible lesion. Carcinoma in situ may be accurately identifiable by cytology.

AGNOR

Human beings have 23 pairs of chromosomes, 22 pairs of autosomes and 1 pair of sex chromosome. Based on location of centromere they are classified as

1. Metacentric
2. Sub metacentric
3. Acrocentric

The acrocentric chromosomes are 13, 14, 15, 21, and 22.

A satellite chromosome is which shows a secondary constriction with chromatic knobs extending from the arms on stalks which are slender .It may also present with negative hetero pyknosis at the ends of chromosome arms.[79]

All the acrocentric chromosomes, showed satellites on their short arms which were associated with nucleolus in the prophase cells. They are loops of DNA involved in transcription of ribosomal RNA (rRNA) genes. It functions in the organization of nucleoli. It is therefore called as nucleolar organizer regions.[75]

Nucleoli are organized at specific regions of specific chromosomes and are best seen at prophase. In this stage, two parts

of the chromosomes appear at diametrically opposite sides of the nucleolus.

In metaphase nucleolus organizer section become very thin, stain poorly, known as secondary constriction. The second constriction may serve as identifying landmark for specific chromosomes.

Acrocentric chromosome approach each other with their satellite more often than randomly expected. Cytogeneticists, started investigating this preferential satellite associations as a cause of chromosomal non disjunctions

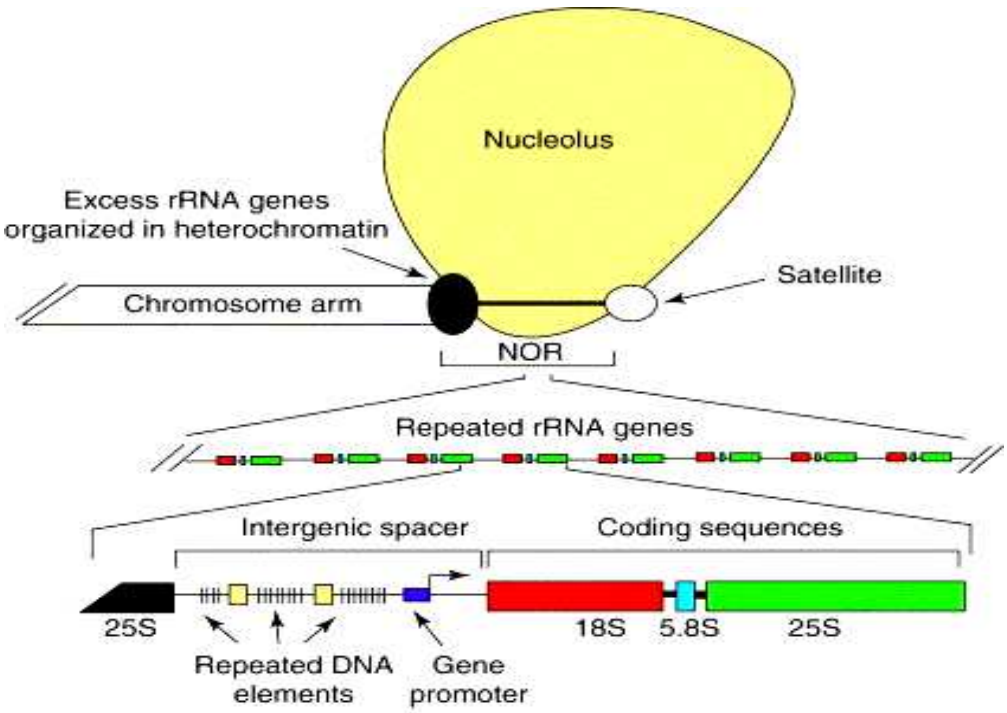


FIGURE 2: ORGANIZATION OF A TYPICAL NUCLEOLAR ORGANIZER REGION[75]

PHYSIOLOGY OF AGNOR'S AND CELLULAR KINETICS:

Ultra structure of the human nucleoli shows three substructures[76].

These are composed of

a) The dense fibrillar component – packed electron dense fibrils.

b) The fibrillar center – composed of loose network of fibrils.

c) The granular component.

The dense fibrillar component has lightly packed electron dense 3-5nm thick fibrils and is the site for processing of ribosomal RNA precursors.

The fibrillar center is the site for the production of ribosomal RNA and it composes topoisomerase I, RNA polymerase I and ribosomal DNA.

The precursor of ribosomes constitutes the granular component.

The behaviour of the tumour may be assessed by determining the proliferation rate. The cell cycle is divided into four phases based on the nuclear chromatin activity. They are S, G1, G2 and G0 phases. There is a short resting phase of the cell undergoing replication at the 'S' phase. Thus, the DNA content at the end of

'S' phase is an indicator of proliferative activity and AgNOR detects the DNA content at this stage.

The size, shape and number of the NORs changes according to nucleolar transcription and are related to the cell cycle. Since nucleolar transcription rate and cell turnover is comparatively high in proliferating cells, assessment of the morphology and quantity of NORs helps in assessing cell proliferation

DEMONSTRATION OF AgNORS:

The NORs can be visualised by various techniques which either demonstrate NOR associated proteins or the ribosomal DNA itself

DEMONSTRATION OF NORs

Reagent	Target
Silver colloid (AgNOR)	NORAPs
Bismuth ions	100K NORAP
Radiolabelled rRNA	rDNA
Antibodies	NORAP epitopes

The simple method among all these in identifying the NORs is the silver staining technique. The structures demonstrated are called AgNORs (Argyrophilic Nucleolar Organizer Regions). This

silver staining technique identifies acidic NORAPs (Nucleolar Organizer Region Associated Proteins) seen in association with the site of RNA transcription.

AgNORs are aggregated tightly within one or two nucleoli in a normal cell, as seen in cytological smears . The number of AgNORs detected depends upon several of these factors. These include stage of the cell cycle, number of NORs bearing acrocentric chromosomes in their karyotype, level of transcriptional activity of the cell.

There is thus a remarkable difference between AgNOR counts in chromosome spreads and those observed in histology sections of the similar cell preparations. Since the AgNORs congregate within a relatively small nucleolus in histological sections, there is a greater difficulty in visualising the individual AgNORs.

In malignant lesions, the AgNORs are dispersed throughout the nucleus to a varying extent, can easily visualised by the cytologists. Therefore 'AgNOR count' in benign and malignant lesions does not denote the absolute number of AgNORs, but it is numerical index of dispersion of AgNORs within the nucleus. Dispersion in itself may reflect the proliferative state of the cell.

Hence the number of visible AgNORs indicates the number of cells in the current phase of transcription.

HISTORY OF STAINING OF AGNOR

By banding techniques Nucleolar organizing regions appeared as achromatic gaps on short arms of specific chromosomes when metaphase preparations were examined. Thus Howell et al felt the need for a technique to differentially stain these important regions. AS – SAT technique by already existing ammonia silver staining where satellites appeared as dark areas above kinetochore of Acrocentric chromosomes.

Goodpasture and Bloom developed a simple silver staining technique in 1995 and they observed silver stained NORs (AgNORs) as black dots on yellow brown chromosome arms. Only transcriptionally active NOR's bind silver

Step I: attachment of silver to the protein nucleolin

Step II: Nucleation of further silver on the bound metal, black appearance is seen

Silver first binds carboxyl groups, then nucleates around disulfide and sulfhydryl groups.

It was postulated by **Buys and Osinga** that the AgNOR reaction involved protein moiety associated with a non-histone.as the reaction occurred extraction of histone .The reduction in silver solution was brought about by-COOH groups of the non-histone proteins containing

The silver deposits were in the form of large aggregates, which were seen at disulfide and sulfhydryl group site. Protein B23, C23 and RNA polymerase-1 are responsible for silver staining of nucleoli.

An improved version of this method was developed by Howell and Black,[77] which required two minutes to perform and used protective colloidal developer (gelatin, de iodized water and formic acid) to control reduction of silver.

Ploton et al further simplified this technique by diminishing the staining temperature from 60^0C to 20^0C by which troublesome background staining was reduced, thereby aiding in better visualization of NOR's.

They applied silver staining technique to paraffin embedded tissue, permitted both a detailed study of nucleoli as well as study of the variation in size and AgNOR count in human tissues which

showed the pathology, in comparison with normal tissue. A pilot study by them suggested that in prostatic carcinoma several AgNOR nuclear dots were seen.

They opined that the easy applicability of AgNOR staining technique to, tissues with pathology, embedded in paraffin permitted the study of silver stainability and assess the relation to grading of cancer tissues and carcinoma in situ. This was modified technique by Ploton et al, adopted by most workers later.[78]

Earlier workers counted 50, 100, and 200 nuclei. It was later found that the mean AgNOR with 100 nuclei obtained was the same as when only 50 were counted.[80]

Crocker et al, described three types of AgNOR configuration

- Solitary rounded argyrophilic structure corresponding to nucleolus. The resting lymphocytes and benign cells are the ones in which these are seen.

- Secondly, as often seen in proliferating cells NOR's could be seen in the nucleus as partially dissociated foci.

- Multiple small, discrete AgNORs may be seen distributed in whole of the nucleus. It is seen as a feature of malignant cells.

Based on this, they advised two ways of counting AgNORs

Firstly, all silver stained structures should be counted, but when lying in groups each cluster should be treated as one structure.

Secondly, where AgNORs can be seen separately within a nucleolus, each AgNOR could be counted as a unit, in addition to the smaller AgNORs seen outside the nucleoli.

Hall et al[81] obtained a linear correlation between Ki-67 immuno reactivity and mean number of NOR in non-Hodgkin's Lymphoma and suggested that NORs were possibly reflection of cellular kinetics.

Smith and Crocker et al[82] showed distinct variation between non neoplastic lesions, benign and malignant lesions **Smith et al**[83] studied the effect of a series of fixatives. Mercurial and dichromatic containing fixatives had detrimental effect while routine 10% normal saline gave good results. Alcohol gave optimal AgNOR staining.

Howat et al[84] found no correlation between AgNOR counts and prognosis in thick cutaneous malignant melanomas

An apparent increase in the mean AgNOR count was noticed in the cells under the following conditions:

- When the cell proliferation was present, the nucleolar dissociation was present, and that the AgNORs were seen throughout the nucleus.
- A defect of the nuclear association results in AgNOR dispersion throughout the nucleus.
- An increase of the AgNOR bearing chromosomes resulting from increased cellular ploidy.

In the benign neoplastic cells AgNORs are aggregated in a condensed small nucleus and hence show only 1-2 AgNOR per nucleus, which is attributed to the difficulty in visualising the individual NORs. While in malignancy, or in conditions of increased cellular proliferation, AgNORs get dispersed throughout the nucleus, enabling to enumerate them more easily. The number of AgNORs detected depends upon several of these factors. These include-

1. The stage of the cell cycle.

2. The number of NORs bearing acrocentric chromosomes in their karyotype

3. The level of transcriptional activity of the cell.

There is thus a remarkable difference between AgNOR counts in chromosome spreads and those observed in histology sections of the similar cell preparations. Since the AgNORs congregate within a relatively small nucleolus in histological sections, there is a greater difficulty in visualising the individual AgNORs.

In malignant lesions, the AgNORs are dispersed throughout the nucleus to a varying extent, enabling them to be easily visualised by the cytologists. Therefore quantification of the AgNORs in interphase nuclei is probably related more to their dispersion throughout the nucleoplasm than to the actual number present in the nucleus. Therefore ‘AgNOR count’ in benign and malignant lesions does not denote the absolute number of AgNORs but it is rather a numerical index of dispersion of AgNORs within the nucleus. This connotes that dispersion in itself may reflect the proliferative state of the cell. Hence the number of visible AgNORs indicates the number of cells in the current phase of transcription.

AgNOR – TECHNICAL ASPECTS

The advantage of the AgNOR staining technique is that, it is a one-step simple staining procedure. It can also be used to demonstrate NORs on processed cytology smears and histology sections. The disadvantage is the time consuming process of counting of the little dots, often associated with inter-observer variations.

This one step silver-staining method consist of a mixture of 50% silver nitrate solution and 1% formic acid in 2gms% of gelatin solution which acts as a colloid stabilizer. These solutions have to be prepared separately and mixed freshly upon use. Cytological smears are incubated in the solution mixture for a period of 45 min and then washed, dehydrated, cleared and mounted for light microscopy examination.

The NORs can be seen as discrete black / brown dots in a pale yellow background and can be enumerated using an oil immersion lens. Counts of 50 neoplastic cells are made, under oil immersion and the results are recorded as a mean number of AgNORs visualised per nucleus.

This technique can be used with image analysis. In this technique the total amount of AgNOR per nucleus is measured.

AgNOR STAINING REACTION & PROBLEMS:

The silver staining technique is based upon the principle that, the silver salts as a result of their high electron charge density and by virtue of their phosphate moieties bind to the acidic NORAPs.

Generally, the AgNOR silver staining method is been run for about 45minutes irrespective of whether cytological smears or histological sections were stained, but recently minor alterations have been put forth to reduce the staining time and also to incorporate internal controls to allow counting of subsidiary AgNOR dots.

First, problem with the usage of any kind of silver staining method is the non-specific silver grain deposits in the background. Usage of clean glassware and very pure deionised water can overcome this background staining. Modifications in the AgNOR staining method have been suggested that can solve this problem of background staining. These are:

1. Usage of an inverted incubation technique, where the slides are inverted into the staining solution. This helps in

maintaining a high degree of contrast between the background and the AgNORs.

2. Glycine blocks both free and reversibly bound aldehyde residues left over by formalin fixation, thereby reducing the background silver precipitation. Pre-incubation with glycine prior to AgNOR staining can reduce the background stain.
3. After completion of the staining procedure immersing the slides in a 10% nitric acid solution can minimise the background stain.
4. Replacement of gelatin by polyethylene glycol as a protective colloidal developer medium.

Second, the intensity of staining varies with variations of the staining time; which over-stained obscures the individually clustered AgNORs within nucleoli, or under-stained renders them too faint .

Third, in the histology sections variations in the thickness of the sections affect the apparent number of AgNORs within the nuclei, thus requiring uniformly thick section of around 3 - 4 μm. Cytology smears doesn't face this issue. Thus AgNOR dot count study on cytological smears is more superior compared to those on histological sections. Cytological smears also show a better

discriminative value of AgNOR dots compared to those on histological sections.

One great advantage of this technique is that, previously stained cytology slides can also be reused for silver staining, thus providing an excellent guide to the diagnosis especially in doubtful cases and when extra-unstained slides are not available.

The major disadvantages are:

1. Inter-observer variation is the major cause of inaccuracy and inconsistency.
2. The counting procedures adopted are usually manual and are prone to subjective variations.
3. Misjudged counts may result due to overlapping of the NORs within the nucleus.
4. The dots of AgNOR in the interphase nuclei may not always correspond to the number of such types in the karyotype of the nucleus[85].

MODIFICATIONS IN THE AgNOR TECHNIQUE:

After the AgNOR technique was first described by Ploton in 1986 it has undergone several modifications with an aim to improve the overall staining quality. Some of the modifications are

1. Combination of Feulgen reaction with modified AgNOR staining technique, which enables the counting of active NORs and also the evaluation of the amount of DNA within the same cell nucleus by the Feulgen reaction.

2. Combination of AgNOR staining technique with cytofluorometric analysis on cell suspensions.

3. The use of AgNOR technique along with automatic image analysis software makes these technique far less prone to subjective errors than the traditional methods[86].

AgNOR - ENUMERATION [87]

The types of Nucleolar Organizer Regions within the nucleus can be categorized into three groups.

The first one is the 'Aggregated AgNOR' which are seen as rounded, solitary structures and corresponds to the nucleolus of the cell, this type is often seen in resting cells and lymphocytes and the

individual NORs cannot be distinguished within the nucleus of these cells.

The second type is the ‘Nucleolar pattern’ which is seen in the nucleus of the proliferating cells and the NORs are seen to be dispersed only within the nucleolus of the cell.

Finally, the third type is the ‘True AgNORs’ that are seen to be dispersed throughout the nucleoplasm and are often seen in highly malignant neoplastic cells. These features can be demonstrated well in the cytological smears.

METHODOLOGY:

There are five methods for enumeration of AgNORs based on their count, morphology and distribution. They are

1. Mean AgNOR count
2. AgNOR proliferative index
3. AgNOR size variation grading
4. AgNOR distribution in the nuclei
5. Subjective AgNOR Pattern Assessment (SAPA)

Mean AgNOR count (mAgNOR):

Mean count of the number of NORs present in the nucleus of the 50 neoplastic cells. mAgNOR value correlates with the mean DNA content of the cells indicating the cell ploidy.

AgNOR proliferative index (pAgNOR):

It is the percentage of neoplastic cells exhibiting more than five NORs within the nucleus of the 50 counted cells. pAgNOR value represents the number of cells in the S-phase fraction.

AgNOR size variation and distribution grading:

In 1991 – 1992 Ahsan et al utilised the criteria of size variation and distribution of AgNORs within the nucleus and demonstrated higher variation score of these parameters in malignant neoplasm compared to the benign counterparts.

TABLE 2 AgNORS SIZE VARIATION GRADING

AgNOR Size Variation	Score
More or less uniform	0
Two different sizes	1+
More than two different sizes (but not those of 3+)	2+
All grades and sizes including too minute to be counted	3+

TABLE 3 AgNOR DISTRIBUTION IN THE NUCLEI

AgNOR distribution – nuclei	Score
Limited to nucleoli	0
Occasional dispersion outside nucleoli	1+
Moderate dispersion outside nucleoli	2+
Widely dispersed throughout the nucleus	3+

Subjective AgNOR Pattern Assessment:

Meehan et al proposed a method for scoring of AgNORs called ‘Subjective AgNOR Pattern Assessment (SAPA), which was based on morphological patterns, variation in the size and shape of the NORs, and whether they are aggregated or scattered

The aim of the present study is to utilize AgNOR staining to identify alteration in Buccal mucosa in case of smokers and compare it with nonsmoker.

In each slide, 50 nuclei were evaluated. The parameter was

- Number of AgNOR dots in the nucleus.

MATERIALS AND METHODS

The study was conducted after clearance from the Institutional ethical committee.

The subjects were 75 male patients who attended ENT and Surgery OPD of Government Stanley Medical College, Chennai.

INCLUSION CRITERIA

Cases were patients who smoked more than 20 cigarettes per day at least for the past 10years or more.

EXCLUSION CRITERIA

Exclusion criteria were applied and the patients rejected were cases of oral ulcer, with premalignant lesion such as leukoplakia, erythroplakia, oral malignancies, and patients with bleeding disorders

CONTROLS

Non smokers were taken as controls.

After a clinical examination and consent, the subjects were requested to gargle for 5 minutes. The lesional areas were wiped off using a sterile gauze piece. Cytological smears were taken from the buccal mucosa. The head of the cytobrush was moistened with

water and firmly held against the mucosa. Then, pressure was applied until bristles curled or tiny bleeding spots were evident. The brush was rolled for 10 full turns. The cytobrush was then rolled on glass slide by applying a continuous motion from one side to other.

PREPARATION OF THE STOCK SOLUTION

Preparation of stock solution A: 1% formic acid solution made by diluting 1ml of formic acid in 99 ml of distilled water. 2 grams of gelatin powder was dissolved in 100ml of 1% formic acid solution. This comprised the Stock Reducing Solution.

Preparation of Silver solution (solution B): A 50% solution of silver nitrate is prepared by dissolving 2 grams of crystalline silver nitrate in 4ml of distilled water.

Preparation of silver colloidal staining solution: 4ml of freshly prepared solution B is mixed with 2ml of stock solution A and used immediately.

METHODS OF STAINING

Smears were layered with silver colloidal mixture. Slides were incubated in a dark room for 40 minutes at room temperature.

The smears were washed in distilled water, dehydrated, cleared and mounted in D.P.X. The stained slides were viewed under 1000x magnification in oil immersion.

AgNOR COUNTING METHOD

Nucleolar organizing regions appeared as clear black dots in the nuclei. The counting was done based on Crocker's method. The maximum number of clearly discernable AgNOR dots was ascertained in 50 nuclei at a magnification of 1000x.

OBSERVATION AND RESULTS

TABLE4-CLASSIFICATION OF THE SUBJECTS INTO GROUPS

Study Groups	Name of Group	Observation	Number of Subjects
Group A	Non - smokers	Agnor count in buccal mucosal cells	50
Group B	Smokers	Agnor count in buccal mucosal cells	25

STATISTICS

Descriptive statistics was done for all data and suitable statistical tests of comparison were done. Continuous variables were analysed with the unpaired t-test and Pearson's R Correlation Test. Statistical significance was taken as $P < 0.05$. The data was analysed using EpiInfo software (7.1.0.6 version; Center for disease control, USA) and Microsoft Excel 2010.

CHART-1 –AGE DISTRIBUTION AMONG THE SUBJECTS

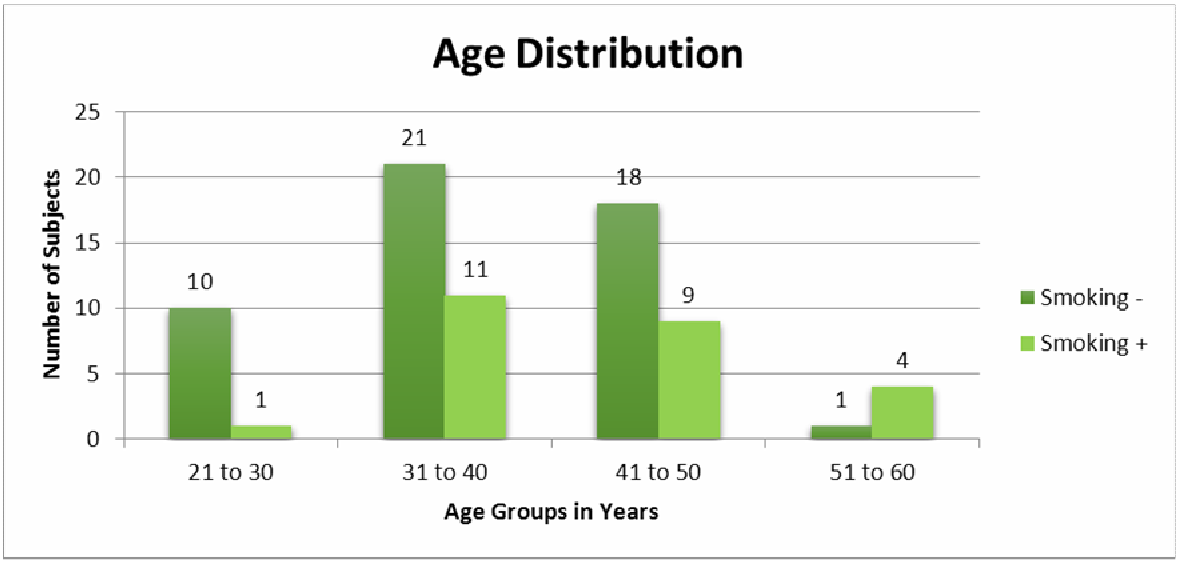


TABLE5-AGE DISTRIBUTION OF THESUBJECTS INVOLVED IN THE STUDY

Age Distribution	Smoking -	%	Smoking +	%
21 to 30	10	20	1	4
31 to 40	21	42	11	44
41 to 50	18	36	9	36
51 to 60	1	2	4	16
Total	50	100	25	100

TABLE6-MEAN AND STANDARD DEVIATION OF THE SUBJECTS

	Smoking -	Smoking +
N	50	25
Mean	39.54	43.24
SD	7.05723	8.222327
P value Unpaired t test	0.061198	

By conventional criteria the association between the groups and age is considered to be not statistically significant since p > 0.05.

CHART2-MEAN AgNOR COUNT AND STANDARD DEVIATION

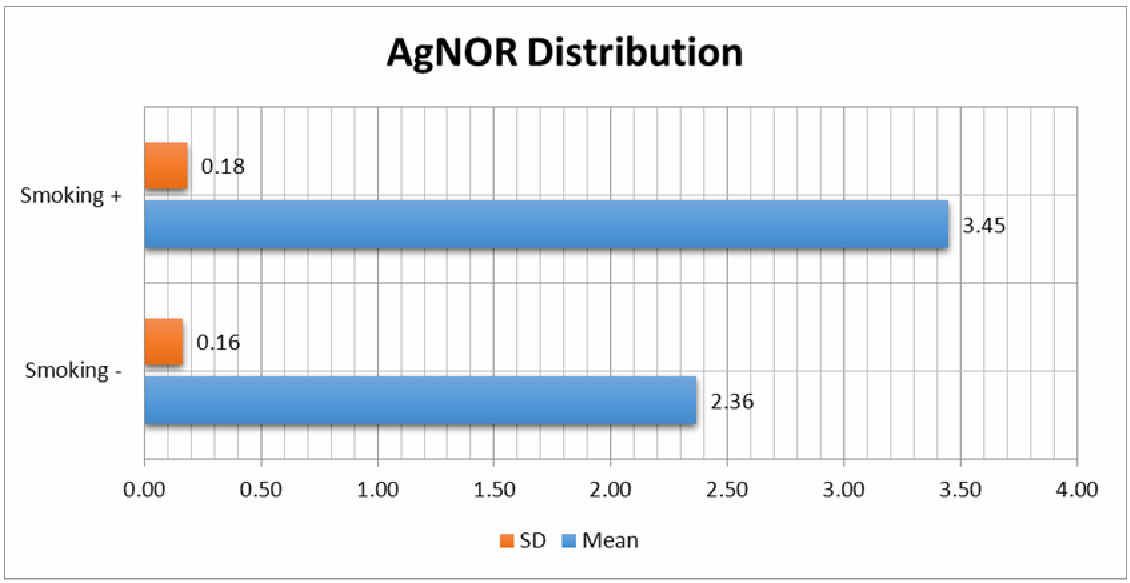


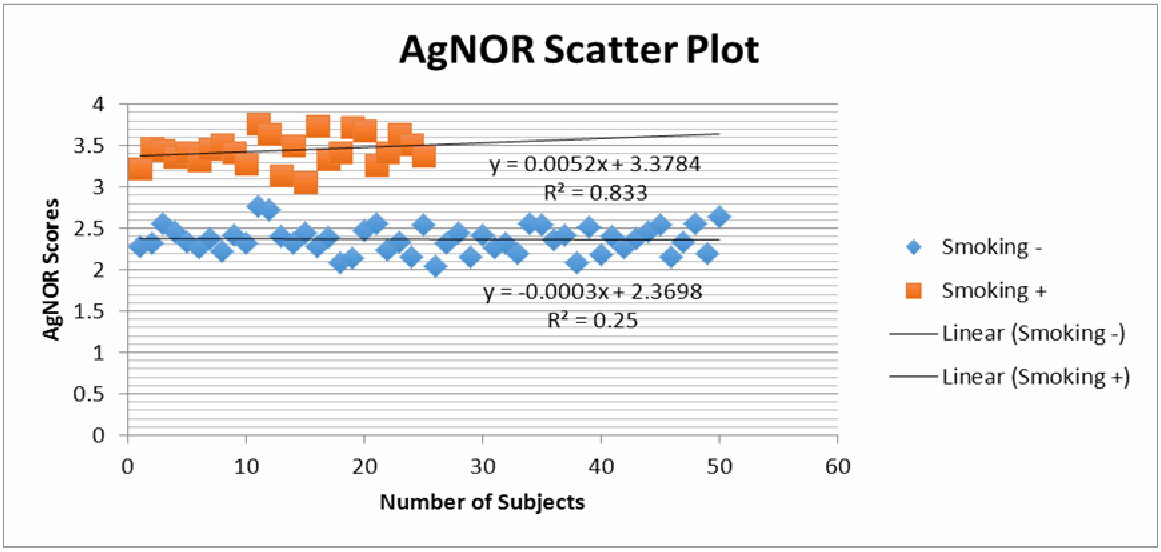
TABLE7-MEAN AgNOR COUNT AND STANDARD DEVIATION

	Smoking -	Smoking +
N	50	25
Mean	2.36	3.45
SD	0.16	0.18
P value Unpaired t test	0.0000	

TABLE 8-CORRELATION BETWEEN SMOKING AND AgNOR COUNT

Pearson's R Correlation	*Smoking* -	*Smoking* +
Smoking -	1	
Smoking +	0.67367954	1

CHART 3 – AgNOR SCATTER PLOT



By conventional criteria the association between the smoking and increase in AgNOR scores in buccal mucosa smears is considered to be statistically significant since $p < 0.05$.

Statistical Significance

This indicates that there is a true difference among treatment groups and the difference is significant. In simple terms, among smokers the AgNOR numbers in buccal mucosa smears are increased to 3.45±0.18 in comparison with non-smokers where AgNOR numbers in buccal mucosa smears are 2.36±0.16 on an average with a p-value of 0.0000 according to Unpaired t-test.

There is a strong positive correlation between smoking and increase in AgNOR numbers in buccal mucosa smears. This is

indicated by the Pearson's R Correlation value of 0.67367954. This means as smoking increases the the AgNOR numbers in buccal mucosa smears increase.

The percentage increase is also explained in the scatter plot. This linear model explains all the variability of the response data around its mean. Since R^2 is 0.833, “the fitted regression equation explains 83.3% of the variation in Y".

Clinical Significance

- The AgNOR numbers in buccal mucosa smears was meaningfully more (46%) among smokers when compared to non-smokers.
- The increase in AgNOR numbers in buccal mucosa smears correlates positively, directly and strongly with the quantum of smoking. This means that the 46% increase in AgNOR numbers in buccal mucosa smears due to smoking is true 67% of times
- This 67% correlation of variation in AgNOR numbers in buccal mucosa smears among smokers is validated 83.3% of times.

CHART4-PROLIFERATIVE AgNOR

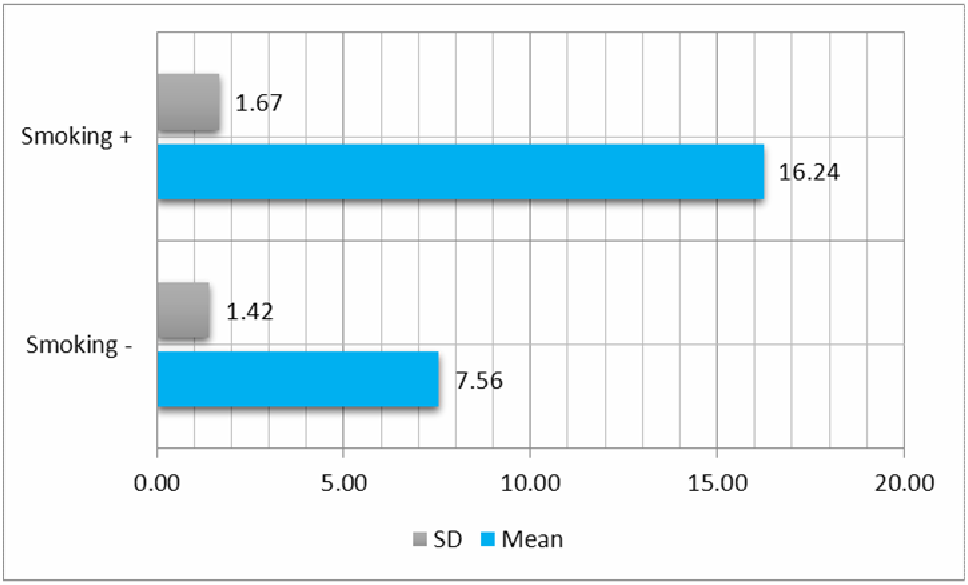


TABLE9- MEAN AND STANDARD DEVIATION OF PROLIFERATIVE AGNOR

Proliferative AgNOR	Non smokers	Smokers
N	50	25
Mean	7.56	16.24
SD	1.42	1.67
P value Unpaired t test	0.0000	

TABLE10 CORRELATION BETWEEN

PROLIFERATIVE AgNOR AND SMOKING

Pearson's r correlation	*smoking -*	*smoking +*
Non smokers	1	
Smokers	0.71999121	1

This indicates that there is a true difference among treatment groups and the difference is significant. In simple terms, among smokers the percentage of cells with Proliferative AgNOR in buccal mucosa smears are increased to 16.24±1.67 in comparison with non-smokers where percentage of cells with PAgNOR Dots in buccal mucosa smears is 7.56±1.42 with a p-value of 0.0000 according to Unpaired t-test.

There is a strong positive correlation between smoking and increase in percentage of cells with PAgNOR(>5) in buccal mucosa smears. This is indicated by the Pearson's R Correlation value of 0. 0.71999121. This means as smoking increases the proliferative AgNORin buccal mucosa smears increase.

CLINICAL SIGNIFICANCE

- The Proliferative AgNOR in buccal mucosa smears was meaningfully more (2.14 times) among smokers when compared to non-smokers.
- The increase in proliferative AgNOR in buccal mucosa smears correlates positively, directly and strongly with smoking. This means that the 2.14 times increase in percentage of cells with>5 AgNOR in buccal mucosa smears due to smoking is true 72% of times

DISCUSSION

Squamous cell carcinoma is the most frequent malignancy of mouth among the ten most common cancers in the world[93]. A strong causal relationship exists between cigarette smoking and development of oral squamous cell carcinoma.

Head and Neck malignancies is one of the most common entity, currently holding sixth position in the worldwide stastistics[94]. Cancer of the oral cavity is more common in the Indian scenario and accounts for one third of cancer cases. 90% of patients are smokers and tobacco chewers[95].

Screening can reduce morbidity and mortality in oral cancers. The most common diagnostic tool is biopsy which is a painful invasive procedure. It can be substituted by exfoliative cytology which is a easy and cheap modality. It can be carried out with a cytobrush and is a useful non- invasive technique for detecting early and suspicious oral lesions.

Exfoliated cells from oral epithelium have been widely used to detect abnormal nuclear and cellular morphology to identify precancerous and cancerous changes. Buccal mucosal cells are

widely affected, as more surface area is exposed to the insult and that the epithelial cells are non- keratinized make them vulnerable to changes due to smoking.

A potent biomarker for measuring the cell activity proliferating potential of cells is AgNOR. AgNOR is associated with acidic, argyrophilic, nonhistone proteins that can be visualized by using silver staining technique.

Silver staining method for identification of NOR in exfoliative cytology is being recently used to know the cellular changes due to smoking. Benign tumor cells exhibit less number of dots per nucleus, roundedness and clustered distribution. Malignant cells show large number of dots per nucleus, irregular shape of AgNOR and scattered distribution.

In our study of AgNOR counting using crockers method was done on 75 samples taken from 50 non -smokers and 25 smokers.

Sampio et.al conducted their study in 1999 ,with 20 smokers and non- smokers. AgNOR staining was carried out. 100 cells were taken into account for counting. The inference was that AgNOR expression was high in smokers. The percentage of cells with

AgNORs numbering more than 5 per nucleus are indicative of high proliferative activity in smokers.

A total of 60 smokers and non- smokers were chosen for evaluation of AgNOR staining of buccal mucosal cells by Jahenshah Salehinejad et.al showed a mean AgNOR higher in smokers than non- smokers. A percentage of cells with 3 or more AgNOR stained dots were determined.

In a study by Ufuk USTA, 116 patients were selected of which 55 were smokers and 61 were nonsmokers. Smokers chosen for this study were found to use 20 filtered cigarettes per day for past 20 years. A total of 200 cells were taken into account for this study and proliferative AgNOR was observed in their study.

55 subjects, 40 patients with history of smoking and tobacco chewing, 10 normal healthy patients and 5 patients with oral SCC were included in a study conducted by anushree sharma and sushmita saxena. AgNOR counting was done in 50 non overlapping cells. The percentage of cells with more than 3 and more than 5 nuclei were seperately calculated..

A study by sethi and shah et.al included 30 smokers and 30 non -smokers and counted 100 cells. They concluded mean AgNOR

was higher in smokers than non -smokers. There was a rise in proliferative AgNOR in smokers.

Sachin Jindal et.al. worked on 25 smokers and 25 non-smokers. 500 cells per slide was counted to note changes. The smokers had the habit for 5 to 10 years and ranged from 5 to 20 cigarettes. The mean AgNOR were on the higher side for smokers in comparison with non- smokers.

TABLE-11-A COMPARISON OF MEAN AGNOR IN VARIOUS STUDIES

S.NO	STUDY	MEAN AgNOR SMOKERS	MEAN AgNOR NONSMOKERS
1.	Sampio et.al[91]	3.4+/-0.54	2.6+/-0.49
2.	Jahenshah sulehinejad et.al[92]	3.6+/-0.43	1.96+/-0.14
3.	Sachin Jindal et.al[93]	4.162+/-0.532	3.352+/-0.760
4.	Sethi and shah et.al[94]	3.79+/-0.480	2.94+/-0.325
5.	UfukUSTA et.al[95]	4.22+/-0.39	3.47+/-0.30
6.	Sharma et.al[96]	3.35+/-0.38	2.91+/-0.23
7.	Present study	2.36+/-0.16	3.45+/-0.18

TABLE12-A COMPARISON OF PROLIFERATIVE AGNOR IN VARIOUS STUDIES

S.NO	STUDY	PROLIFERATIVE AgNOR (>5) SMOKERS	PROLIFERATIVE AgNOR(>5) NONSMOKERS
1.	Sampio et.al	20%	6%
3.	Sethi and shah et.al	26.5%	11.7%
4.	UfukUSTAet.al	36.8%	14.6%
5.	Present study	16.24%	7.56%

Zimmerman and Zimmerman observed that age smoking habit, oral and systemic disease modified the degree of keratinization of oral mucosa at certain sites.

Study conducted by Sampios included patients ranging from 41 to 77 with a mean age of 57 in the non-smoking group and 40 to76 years with a mean of 53 years in the smoking group. AgNOR expression was not found to be influenced by age factor.

In the present study, subjects ranging from 28 to 58 years were taken. The mean age for smokers was 43.24 years and for non-

smokers was 39.54 years. There was no significant correlation between age and the expression of AgNOR in this study.

According to world health organization, over 1 billion people are associated with tobacco smoking. More than 20 cigarettes per day increases the risk of oral cancer. It has been observed that more than 4 to 5 million deaths per year occurs due to smoking. This number is estimated to increase to 10 million by 2030. Second hand smoking may also increase a person's risk of head and neck cancer.

Smokers taken up for the study by UfukUSTA were found to smoke 20 filtered cigarettes per day for past 20 years.

Jahenshah included smokers using a minimum of 20 cigarettes per day for 10 years.

In our present study group, samples were taken from patients who were chronic smokers for more than 10 years and smoked a minimum of 20 cigarettes per day.

Poor oral hygiene increases the risk of oral cancers. Poor dental health or prolonged irritation from ill- fitting dentures in tobacco smokers contributes to oral cancer.

Poor nutrition and weakened immune system also predisposes to higher risk of developing oral cancer. In a study conducted by Lynne Adikins observed that poor oral hygiene leads to more than 50% chance of occurrence of oral HPV infection.

Castro Sampio and Jahenshah observed that there were no oral or systemic disease in their patients. 4 patients were observed to present with poor oral hygiene in our study and the AgNOR count was observed to be high in such patients .

Mean AgNOR count is a valuable criterion for defining objective parameters for the diagnosis of premalignant and malignant lesions of oral cavity.

Remmerbach et.al inferred a mean AgNOR of 2.31+/-0.7 in non- smokers and 3.39+/-0.4 in smokers. A similar observation was made by Mao et.al. He observed a mean AgNOR of 2.44+/-0.3 in non- smokers and 4.69+/-0.72 in case of oral cancer.

The mean AgNOR in smokers was found to be 3.79+/-0.48 and in non- smokers it was 2.94 +/- 0.325 in a study conducted by sethi and shah et.al.

Studies by Jahenshah et.al revealed a mean AgNOR of 1.96+/-0.14 in non- smokers and 3.6+/- 0.43 in smokers.

Studies by Chattopadhyaya et.al showed a mean AgNOR of 2.732 +/- 0.236 in non-smokers and 3.372 +/- 0.375 in case of smokers.

Xin Xie made an observation of mean AgNOR of 2.3+/- 0.4 in case of non-smokers and 3.8+/- 0.8 in case of dysplasia.

AgNOR expression in the study by sachin Jindal showed a mean AgNOR of 4.162+/- 0.53 and 3.352+/-0.76.

Studies by sharma et.al showed a mean AgNOR of 3.35+ /- 0.38 in smokers and 2.91+/- 0.23 in non smokers.

The observations made by UfukUSTAet.al showed a mean AgNOR of 4.22+/- 0.39 in case of smokers and 3.47 +/- 0.3 in case of non-smokers.

In comparison with other studies the present study showed a similarity to the studies of Mao et.al, Remmerbach et.al, Sampio et.al,and Sharma et.al and Jahenshah et.al, Xin xie et.al, while higher mean AgNOR value was observed in studies conducted by Sachin Jindal et.al and UfukUSTA et.al.

Proliferative AgNOR is one another parameter which is a reflection of the proliferative activity of the cell. It can be

calculated by enumerating the number of cells with greater than 5 AgNOR dots per cell. It can also be done for greater than 3 AgNOR dots per cell.

The proliferative AgNOR in Sampio et.al study was 20% in smokers and 6% in non smokers. In the study by Sethi and Shah et.al it was 26.5% in smokers and 11.7% in nonsmokers.

The present study showed a proliferative AgNOR of 16.24 % and 7.56% in smokers and non-smokers respectively.

SUMMARY

This prospective study was carried out at government Stanley medical college over a period of 12 months from 2013 to 2014. AgNOR expression was observed in both smokers and non-smokers.

This study has helped us in to make the following interpretation.

1. 50 Non overlapping cells were taken for the study in each smear.

2. Age did not have a significant impact in the expression of AgNOR per nucleus.

3. High mean AgNOR count was seen in smokers for more than 10 years and who used a minimm of 20 cigarettes per day.

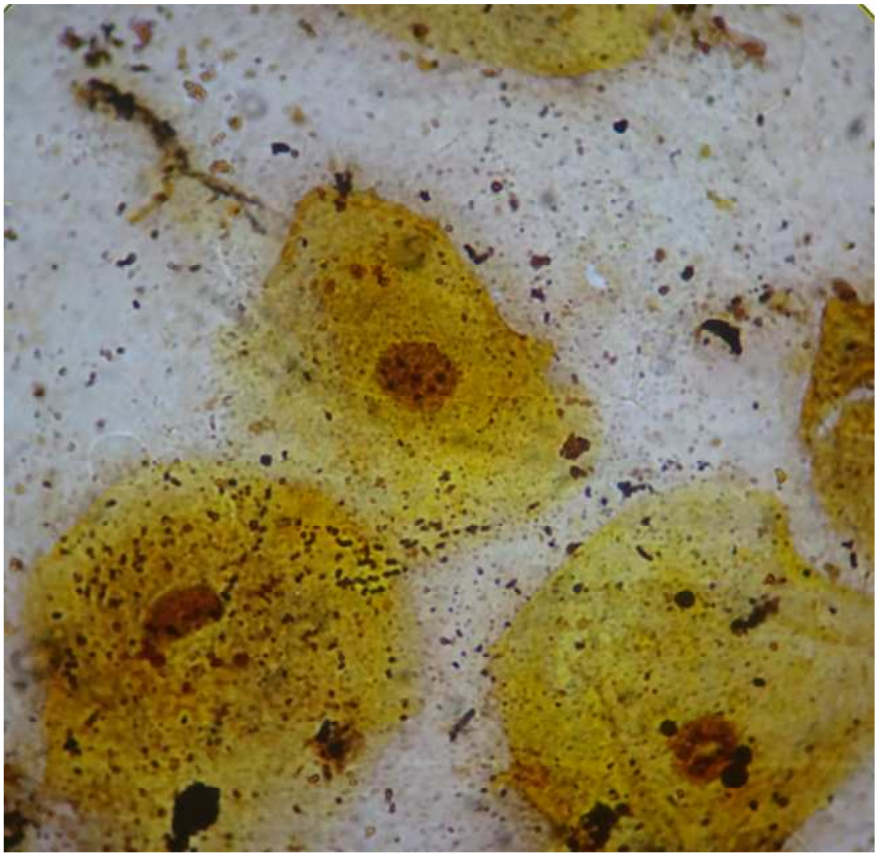
4. The mean AgNOR count in smokers was 3.45+/-0.18and in non smokers was 2.36+/-0.16

5. The proliferative AgNOR was 16.24% in smokers and 7.56% in non smokers.

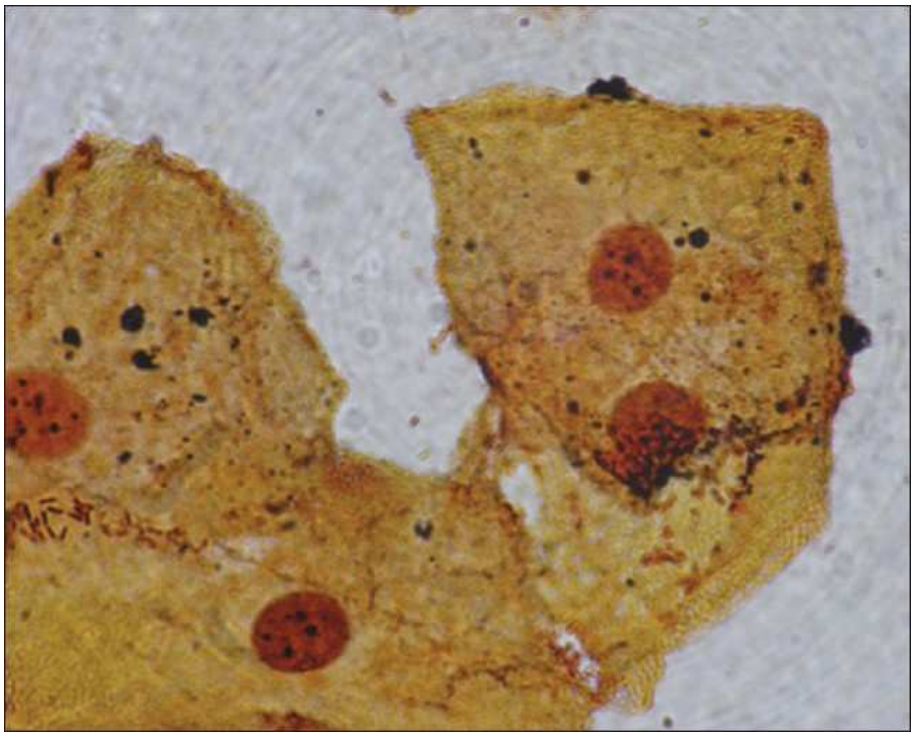
CONCLUSION

This study supports and extends the view that cytological changes seen in oral mucosa due to smoking detected by AgNOR application serves as a useful, simple, preliminary diagnostic aid in observing changes in apparently normal buccal mucosa and assessing it as a risk factor in progression to cancer.

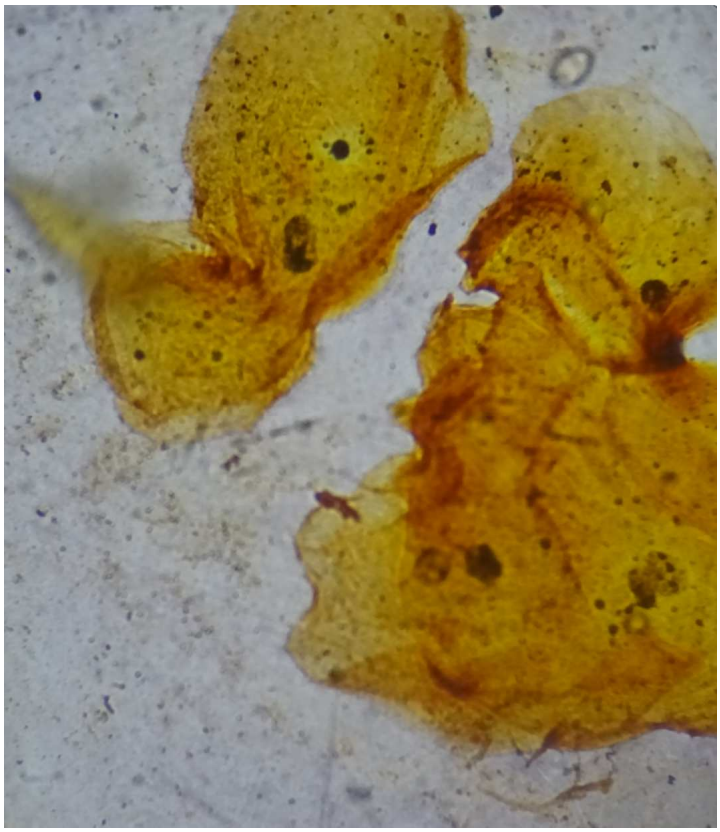
It also supports the expectation that AgNOR as a biomarker for proliferative changes in normal buccal mucosa, will prove beneficial in smoking cessation counselling.



SMEAR SHOWS 4 TO 5 AgNOR DOTS PER NUCLEUS (X 1000) IN SMOKERS



SMEAR SHOWS 5 TO 6 AgNOR DOTS PER NUCLEUS (X 1000) IN SMOKERS



LOW POWER VIEW O F AGNBOR

SMEAR SHOWS 1 TO 2 AgNOR DOTS PER NUCLEUS OF EVEN SIZE AND SHAPE (X 1000) IN NON SMOKERS

SMEAR SHOWS 3 TO 4 AgNOR DOTS PER NUCLEUS OF EVEN SIZE AND SHAPE (X 1000) IN NON SMOKERS

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PROFORMA

Name	:
Age	:
IP No	:
Pathology ID Number	:
Complaints	:
Past History	:
Personal History	:
Family History	:
General and local examination	:
Investigation report	:
Treatment History	:

INSTITUTIONAL ETHICAL COMMITTEE, STANLEY MEDICAL COLLEGE, CHENNAI-1

Title of the Work : Alteration in Buccal Mucosal Cells due to the effect of Tobacco and Alcohol by assessing the silver stained nucleolar organizing regions.

Principal Investigator : Dr. K Shanmugam

Designation : PG in MD (Pathology)

Department : Department of Pathology
Government Stanley Medical College,
Chennai-01

The request for an approval from the Institutional Ethical Committee (IEC) was considered on the IEC meeting held on 10.01.2014 at the Council Hall, Stanley Medical College, Chennai-1 at 2PM

The members of the Committee, the secretary and the Chairman are pleased to approve the proposed work mentioned above, submitted by the principal investigator.

The Principal investigator and their team are directed to adhere to the guidelines given below:

1. You should inform the IEC in case of changes in study procedure, site investigator investigation or guide or any other changes.
2. You should not deviate from the area of the work for which you applied for ethical clearance.
3. You should inform the IEC immediately, in case of any adverse events or serious adverse reaction.
4. You should abide to the rules and regulation of the institution(s).
5. You should complete the work within the specified period and if any extension of time is required, you should apply for permission again and do the work.
6. You should submit the summary of the work to the ethical committee on completion of the work.

MEMBER SECRETARY,
IEC, SMC, CHENNAI

சுயஒப்புதல் படிவம்

வாயின் உட்புறத்திலிருந்து எடுக்கபட்ட செல்களின் பகுப்பாய்வு

ஆராய்ச்சிநிலையம் : நோய்கருறியியல்த்துறை

ஸ்ட்டான்லீ மருத்துவக்கல்லூரி,
சென்னை - 600 001.

பங்கு பெறுபவரின் பெயர் :

பங்கு பெறுபவரின் எண் :

மருத்துவ ஆய்வின்விவரங்கள் எனக்குவிளக்கப்பட்டுள்ளது.
எனது ஆய்வுப்ற்றியசந்தேகங்களை கேட்கவும் அதற்கானதகுந்த விளக்கங்களை பெறுவும் வாய்ப்பளிக்கபட்டது.

நான் எனது வாயின் உட்புறத்திலிருந்து எடுக்கபட்ட செல்களை பயன்படுத்த இச்சையாகசம்மதிக்கிறேன். எக்காரணத்தாலும் எந்த கட்டத்திலும் எந்தசாட்சிகளுக்கும் உட்படாமல் நான் இவ்வாய்வில் விலகிக்கொள்ளலாம் என்றும் அறிந்து கொண்டேன்.

இந்த ஆய்வுமூலம் கிடைக்கும் தகவல் களையும் பரிசோதனை முடிவுகளையும் மருத்துவர்மேற் கொள்ளும் ஆய்வில்பயன்படுத்தி கொள்ளவும் அதை பிரசுரிக்கவும் நான் முழுமனதுடன் சம்மதிக்கிறேன்.

எனக்கு கொடுக்கப்பட்ட அறிவுரைகளின்படி நடந்து கொள்வதுடன் இந்த ஆய்வுமேற் கொள்ளும்மருத்துவருக்கு உண்மையுடன் இருப்பேன் என்றும் உறுதி அளிக்கிறேன்.

பங்குபெறுபவரின்கைஒப்பம் : இடம் :

நாள் :

கட்டைவிரல் ஒப்பம் :

பங்குபெறுபவரின் பெயர்மட்டும்விலாசம் :

தகவல்படிவம்

தங்களுக்குசெய்த வாயின் உட்புறத்திலிருந்து எடுக்கபட்ட செல்களின் பகுப்பாய்வு ஆராய்ச்சிமூலம்தங்களுக்கு பிரச்சனை உள்ளதுகண்டுபிடிக்கப்பட்டுள்ளது அதற்கானகாரணம் அறியஆய்வு மேற்கோள்ப்படஉள்ளது. இதில் தங்களது நோய் குறித்த விவரங்கள் இதர ஆராய்ச்சி முடிவுகளை தங்கள் சம்மதத்துடன் இவ்வாய்வில் பயன்படுத்தவிரும்புகிறோம். பின்னாளில் மீண்டும் ஆய்வில் பங்கேற்க்கவும் தங்கள் முழுசம்மதம் பெற்ற பின்னர் மட்டும் மேற்கோள்ளப்படும்.

தாங்கள் விரும்பினால் இவ்வாய்வில் இருந்து எப்பொழுது வேண்டுமானாலும் விலகிக் கொள்ளலாம்.

இவ்வாய்வில்மூலம் கிடைக்கும் தகவல்களும், பரிசோதனை முடிவுகளும் தங்களின் ஒப்புதல் மூலம்மட்டுமே ஆய்வில் பயன்படுத்தப்படும்.

ஆய்வாளரின்கைஒப்பம்: இடம்

நாள்:

ஆய்வாளரின்பெயர்:

INTRODUCTION

Medical science has made considerable progress with respect to infectious diseases. In case of carcinoma, there is a lot to be achieved, although a good deal of innovations and lifesaving therapies have been discovered. In case of oral cancers, it begins with use of tobacco. It is the powdered leaf of a plant which was used in a Y shaped piece of cone called "Tobago".

Malignancy of the oral cavity is the sixth most common malignancy worldwide.[1] It has a dismal 5-year survival, except when it is detected in early stages. The established method for diagnosis is by biopsy, which is carried out only when patient is symptomatic. Hence it is of little value in detecting at an early stage and preventing the progression.

In Indian subcontinent, oral cancer is one of the most common forms of cancer. Mostly they are squamous cell carcinomas. It is a major public health

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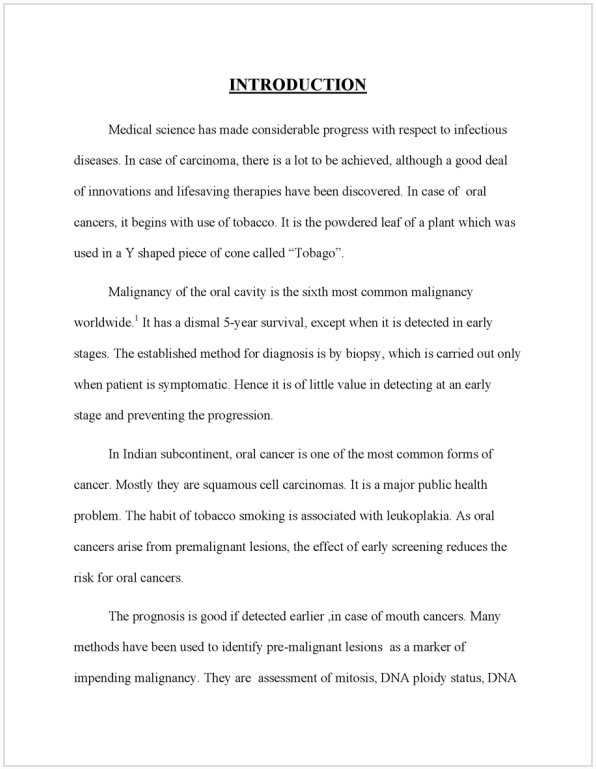

INTRODUCTION

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In Indian subcontinent, oral cancer is one of the most common forms of cancer. Mostly they are squamous cell carcinomas. It is a major public health problem. The habit of tobacco smoking is associated with leukoplakia. As oral cancers arise from premalignant lesions, the effect of early screening reduces the risk for oral cancers.

The prognosis is good if detected earlier ,in case of mouth cancers. Many methods have been used to identify pre-malignant lesions as a marker of impending malignancy. They are assessment of mitosis, DNA ploidy status, DNA

SL.NO.	AGE/SEX	SM/NSM	mAgNOR	pAgNOR
1	30/M	NSM	2.28	4
2	3O/M	NSM	2.32	3
3	45/M	NSM	2.56	5
4	35/M	NSM	2.46	4
5	50/M	SM	3.22	8
6	28/M	NSM	2.34	3
7	27/M	NSM	2.26	4
8	48/M	SM	3.48	9
9	50/M	NSM	2.38	4
10	29/M	NSM	2.22	3
11	35/M	SM	3.44	8
12	31/M	NSM	2.42	3
13	40/M	NSM	2.32	4
14	44/M	SM	3.36	7
15	38/M	SM	3.42	8
16	30/M	SM	3.32	7
17	35/M	SM	3.46	9
18	31/M	SM	3.52	10
19	37/M	SM	3.42	9
20	43/M	SM	3.28	8
21	58/M	SM	3.76	9
22	50/M	SM	3.64	8
23	36/M	SM	3.14	7
24	50/M	SM	3.5	8
25	40/M	SM	3.06	7
26	53/M	SM	3.74	9
27	40/M	NSM	2.76	5
28	46/M	NSM	2.72	4
29	50/M	NSM	2.4	4
30	46/M	NSM	2.32	3
31	38/M	NSM	2.44	4
32	29/M	NSM	2.28	3
33	47/M	NSM	2.39	4
34	28/M	NSM	2.08	3
35	32/M	NSM	2.14	3
36	33/M	SM	3.34	8
37	40/M	SM	3.42	8
38	50/M	SM	3.72	9
39	38/M	SM	3.68	8
40	48/M	NSM	2.48	4
41	30/M	NSM	2.56	3
42	35/M	NSM	2.24	4
43	30/M	NSM	2.34	4
44	35/M	SM	3.26	7
45	50/M	NSM	2.16	5
46	54/M	SM	3.42	7
47	53/M	SM	3.64	8
48	50/M	SM	3.52	9
49	40/M	NSM	2.54	4

50	50/M	SM	3.38	8
51	43/M	NSM	2.04	4
52	40/M	NSM	2.32	5
53	45/M	NSM	2.44	5
54	40/M	NSM	2.16	4
55	34/M	NSM	2.42	4
56	35/M	NSM	2.26	3
57	50/M	NSM	2.32	3
58	40/M	NSM	2.2	3
59	44/M	NSM	2.56	5
60	34/M	NSM	2.54	4
61	36/M	NSM	2.36	3
62	41/M	NSM	2.42	3
63	48/M	NSM	2.08	3
64	45/M	NSM	2.52	4
65	50/M	NSM	2.18	3
66	54/M	NSM	2.4	3
67	38/M	NSM	2.26	4
68	34/M	NSM	2.38	5
69	42/M	NSM	2.46	5
70	44/M	NSM	2.54	4
71	40/M	NSM	2.16	3
72	36/M	NSM	2.34	4
73	33/M	NSM	2.56	4
74	30/M	NSM	2.2	3
75	37/M	NSM	2.64	4